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Genetic diversity and structure of calanoid copepods: Molecular evolutionary patterns in coastal estuaries (Acartia tonsa) and the open ocean (Calanus spp)

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GENETIC DIVERSITY AND STRUCTURE OF CALANOID COPEPODS: MOLECULAR EVOLUTIONARY PATTERNS IN COASTAL ESTUARIES (*ACARTIA TONSA*) AND THE OPEN OCEAN (*CALANUS SPP.*)

BY

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DEDICATION

This work is dedicated to my wife, Kimberly, for her unyielding love and support through what, at times, must have seemed an endless journey. To my children, Dolan, Kaleigh, and Abby for their inspiration and patience. And to my parents, for providing me with the foundation upon which my life is built.

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ABSTRACT

GENETIC DIVERSITY AND STRUCTURE OF CALANOID COPEPODS: MOLECULAR EVOLUTIONARY PATTERNS IN COASTAL ESTUARIES (*ACARTIA TONSA*) AND THE OPEN OCEAN (*CALANUS SPP.*)

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Calanoid copepods are an important part of marine and estuarine ecosystems. However, it has been difficult to study their life histories, population structure, and evolution because they share a conserved morphology that complicates species identification. A primary focus of this study was the genetic and physiological variation of the calanoid copepod *Acartia tonsa* from four estuaries along the East Coast of the USA (Great Bay, NH, Buzzards Bay, MA, Narragansett Bay, RI, and Beaufort Inlet, NC). Based on DNA sequence variation for the mitochondrial cytochrome oxidase I (mtCOI) gene, significant population structure was observed between all pairs of estuarine populations ($\Phi_{ST} > 0.9$, $p < 0.0001$), except for those of two neighboring estuaries, Buzzards Bay and Narragansett Bay. Based on amplified fragment length polymorphism (AFLP) markers, significant population structure was observed between Buzzards Bay and Narragansett Bay ($\Theta^B = 0.042$, 95% CI: 0.024 – 0.065). Individuals from the four estuarine populations were shown to interbreed with each other in reciprocal crosses in laboratory cultures. Crosses

between individuals from Great Bay, NH and the other populations failed to produce fertile offspring. Thus, according to both the biological and evolutionary species concepts, the Great Bay, NH population of *A. tonsa* should be considered to be a different species from the Buzzards Bay, MA; Narragansett Bay, RI; and Beaufort Inlet, NC populations. Comparisons of physiological responses to high and low temperatures of Great Bay and Beaufort Inlet populations yielded evidence of significantly different responses to temperature extremes. Attempts to induce females to lay diapause egg by exposing them to low temperatures and short photoperiods produced only quiescent eggs. It was concluded that the currently identified group of *A. tonsa* contains at least two cryptic species that are genetically diverse, potentially evolutionarily and taxonomically distinct, and morphologically identical. The second focus of this study was a parallel analysis of nine species of *Calanus* based on DNA sequence variation of mtCOI. There was significant genetic divergence between all species, which was used to reconstruct the phylogenetic relationship among the species. The molecular phylogeny was in good agreement with hypotheses of evolutionary relationships based on morphological characters. The DNA sequences were also used to develop a PCR-based molecular protocol to rapidly identify four of the species of *Calanus* with very similar morphologies and overlapping geographic ranges. Considered together, the results of these studies showed that the conserved morphology of the calanoid copepods harbor a large amount of genetic diversity, which can be used to identify species and reconstruct their evolutionary relationships. These studies have also indicated that the true diversity of calanoid copepods is yet to be discovered.

CHAPTER I

INTRODUCTION

Copepods, a subclass of the phylum Crustacea, may be the most numerous multicellular organisms on earth (Mauchline 1998), yet their true diversity is poorly understood (Humes 1994). Currently, there are approximately 11,500 known species of copepods divided among about 200 families, however some estimates suggest that this number represents only 15% of their actual diversity (Humes 1994).

The order Calanoida is of particular importance to marine and estuarine ecosystems because they are a dominant primary consumer and are consumed by everything from invertebrates to fish larvae to whales (Mauchline 1998). However, their conserved morphology has made taxonomic identification and classification difficult (Bucklin et al. 1997; Bucklin et al. 1999; Bucklin et al. 2003). This has made it difficult to study the life histories, population structure, diversity, evolutionary histories, species interactions, and ecosystem dynamics of these important organisms in a wide range of environments, from coastal estuaries to the open ocean.

This study has a primary focus - on the calanoid copepod *Acartia tonsa*. *Acartia tonsa* was originally described by Dana (Dana 1849) from samples collected at Port Jackson, Australia. Giesbrecht (Giesbrecht 1892) latter identified *A. tonsa* off the coast of Chile and Peru. *Acartia tonsa* was identified in the USA at Woods Hole, Massachusetts

(Wheeler 1900), San Diego, California (Ritter 1904), and Narragansett Bay, Rhode Island (Williams 1906). It has subsequently been described in estuaries or coastal regions in the western Atlantic seaboard from Nova Scotia to Argentina, in the eastern Pacific from northern California to Central America, and in estuaries along the east Atlantic seaboard from Norway to Spain and the Mediterranean Sea (Heinle 1966a; Uye and Fleminger 1976; Paffenhofer and Stearns 1988; Sabatini 1990; Tester and Turner 1991; Garmew et al. 1994;Table 1-1). It is unlikely that the copepod originally described by Dana in Australia is conspecific with the copepod referred to as *A. tonsa* today, which is morphologically more similar to the samples described by Giesbrecht (Ferrari 1989). European populations may have been derived from American populations via transport in ship ballast water (Remy 1927). *Acartia tonsa* around the world likely comprises native populations, transported populations, and/or misidentified populations, and it can be difficult to distinguish among these.

Populations of *A. tonsa* along the East Coast of the USA south of Cape May, New Jersey are present in the water column year round, while northern populations over-winter as dormant eggs and are replaced in the water column by *A. hudsonica* (Zillioux and Gonzalez 1972; McAlice 1981b; Durbin et al. 1990). McAlice (McAlice 1981b) has theorized that *A. tonsa* invaded areas north of Cape Cod approximately 7,000 to 9,500 years ago and became isolated from each other and from the southern populations 2,000 to 5,000 years ago when they were forced into estuaries by changing sea levels.

Acartia tonsa is an ideal model estuarine organism. It is easily cultured (Heinle 1969; Stottrup et al. 1986), has a short generation time (about 20 days at 18°C; Stottrup et al. 1986), produces large broods (an average of 18/day per female at 18°C with a total

Location	Country	State/Region	Reference
	North America, East Coast		
New Burswick Canada	Canada		(Ketchum et al. 1952)
Inner Bay of Fundy	Canada		(Daborn and Brylinsky 1981)
Saint John River	Canada	New Brunswick	(Carter and Dadswell 1983)
Shediac Bay	Canada	New Brunswick	(Citarella 1982)
Trinity Bay	Canada	Newfoundland	(Napolitano et al. 1997)
Northumberland Strait	Canada	North West Atlantic	(Citarella 1999)
Annapolis River	Canada	Nova Scotia	(Corkett 1981)
Gulf of Maine; Gulf of St Lawrence	USA/ Canada	Maine	(McAlice 1981a)
Great Bay	USA	New Hampshire	(Caudill and Bucklin 2004)
Barnstable Harbot	USA	Massachusetts	(Ketchum 1954)
Boston Harbor and Cape Cod Bay	USA	Massachusetts	(Turner 1994)
Cape Cod Canal, Buzzard Bay, and Cape Cod Bay	USA	Massachusetts	(Anraku 1964)
Narragansett Bay	USA	Rhode Island	(Williams 1906)
Long Island Sound	USA	New York/ Connecticut	(Conover 1959)
Raritan Bay	USA	New Jersey	(Jeffries 1962)
Navesink and Shrewsbury Rivers	USA	New Jersey	(Shaheen and Steimle 1995)
Patuxent River Estuary	USA	Maryland	(Heinle 1966b)
Chesapeake Bay	USA	Maryland/Virginia	(Storms and Taylor 1972)
York River	USA	Virginia	(Jeffries 1962)
Newport River Estuary	USA	North Carolina	(Stearns 1984)
North Inlet	USA	South Carolina	(Lonsdale and Coull 1977)
Georgia Estuaries	USA	Georgia	(Stickney and Knowles 1975)
Biscayne Bay	USA	Florida	(Woodmansee 1958)
Florida Bay	USA	Florida	(Kleppel and Hazzard 2000)
Fort Myers Beach (Gulf side)	USA	Florida	(Gunter et al. 1948)
Phosphorescent Bay	USA	Puerto Rico	(Rios Jara 1998)
Northern Gulf of Mexico	USA		(Chen and Marcus 1997)
Mississippi	USA	Mississippi	(Perry and Christmas 1973)
Mouth of Mississippi River	USA	Mississippi	(Turner 1984)
Lake Pontchartrain	USA	Louisiana	(Darnell 1961)
Louisiana	USA	Louisiana	(Park et al. 1989)
Calcasieu estuary	USA	Louisiana	(Vecchione 1991)
Corpus Christi, Copano-Aransas Bay System	USA	Texas	(Kalke 1980)
East Lagoon	USA	Texas	(Ambler 1983)
Laguna Madre	USA	Texas	(Buskey and Stockwell 1993)
San Antonio Bay	USA	Texas	(Matthews 1981)
Chetumal Bay	Mexico		(Suarez 1994)
Bojorquez lagoon	Mexico	Quintana Roo	(Alvarez-Cadena et al. 1996)
Urias Estuary	Mexico	Sinaloa	(Alvarez and Cortes 1990)
Lagoon complex of Chelem	Mexico	Yucatan	(Escamilla et al. 2001)
South Sinaloa	Mexico		(Hendrickx and Sanchez Osuna 1983)

Table 1-1

Location	Country	State/Region	Reference
	South America, East Coast		
La Habana Bay	Cuba		(Diaz Zaballa and Gaudy 1996)
Saco da Mangueira	Brazil	Lagoa dos Patos	(Montu and Gloeden 1982)
Cananeia Lagoon	Brazil	Neotropical	(Ara 2001)
Patos Lagoon Estuary	Brazil	southern	(Abreu et al. 1994)
Solis Grande	Uruguay		(Gomez et al. 2000)
Valdes Peninsula	Argentina		(Vinas 1991)
Bahia Blanca Bay	Argentina	Buenos Aries	(Hoffmeyer 1987)
	North America, West Coast		
Alviso Pond, San Francisco Bay	USA	California	(Carpelan 1957)
La Jolla	USA	California	(Esterly 1920)
San Diego	USA	California	(Ritter 1904)
Los Angeles Harbor	USA	California	(McConaugha 1976b)
South Pedro Bay	USA	California	(McConaugha 1976a)
Tampa Bay Florida	USA	California	(Hopkins 1977)
San Ramon Beach	USA	California	(Jimenez 1989)
Southern California: Cape Medocino to Magdalena (Baja California)	USA and Mexico	California	(Fleminger 1964)
Bahia Magdalena	Mexico		(Palomares and Gomez 1996)
Lagoon of Yavaros	Mexico	Sonora	(Turcott 1977)
	South America, West Coast		
Arauco Guld	Chile		(Bernal et al. 1986)
North of the Humboldt Current region off Chile	Chile		(Escribano and Hidalgo 2000)
Central Chile	Chile		(Peterson et al. 1988)
area between Los Vilos and Valparaiso	Chile		(Rosales and Sepulveda 1992)
Mejillones Bay	Chile	Northern	(Hidalgo and Escribano 2001)
Between Valparaiso, Chile and Callao, Peru	Chile and Peru		(Giesbrecht 1892)
Cabo Nazca	Peru		(Smith et al. 1981)
	Europe, Mediterranean		
Berre Lagoon	France	Mediterranean	(Gaudy and Pagano 1987)
Venice Lagoon (Northern Adriatic Sea)	Italy		(Comaschi et al. 1994)
Po Delta, Sacca di Goro	Italy	Northern Adriatic Sea	(Sei et al. 1996)
Gulf of Riga, Baltic Sea	Estonia/Latvia		(Berzins 1940)
Black Sea	Mixed (South Eastern Europe)		(Belmonte et al. 1994)
Azov Seas	Ukraine	Northern Black Sea	(Prusova et al. 2002)
Caspian and Azov Seas	Russia		(Prusova et al. 2002)
Warri River	Nigeria		(Oronsaye 1993)

Table 1-1, continued

Location	Country	State/Region	Reference
	Europe, Northern		
Horsea Lake, England (man made)	UK		(Lucas 1996)
River Tyne Estuary	UK		(Matthiessen et al. 1998)
Solent-Southampton Water estuarine system	UK		(Castro 2001)
Buchan area	UK	Scotland	(Kiorboe and Johansen 1986)
South Coast of Finland	Finland	South Coast	(Vorstman 1946)
Copenhagen Harbor	Sweden		(Andersen et al. 1998)
Balsfjorden	Norway	Northern	(Norrbin et al. 1990)
Vistula Gulf of the Baltic Sea	Poland		(Naumenko 2000)
Pomeranian Bay (Southern Baltic Sea)	Germany/ Poland		(Schmidt et al. 1998)
Southern Baltic Bay	Germany/ Poland		(Thiel 1996)
Northern Wadden Sea of Sylt	Germany		(Martens 1981)
Kiel Bay and adjacent waters (southwestern Baltic)	Germany		(Madhupratap et al. 1996)
Darss-Zingst Estuary, Southern Baltic	Germany		(Arndt and Schnese 1986)
Kiel Bight	Germany		(Voss 1991)
Schlei Fjord, Western Baltic	Germany		(Plaga 1983)
Ems estuary (North Sea)	Germany/ Netherlands		(Baretta and Malschaert 1988)
Netherland	Netherlands		(Redeke 1934)
Westerschelde and the Gironde	Netherlands		(Tackx et al. 1995)
Oosterschelde Estuary	Netherlands	South West	(Bakker et al. 1988)
Sluice-dock at Ostend Belgium	Belgium		(Tackx and Polk 1982)
Arcachon Bay	France		(Vincent et al. 2002)
Harbour of Dunkirk	France		(Brylinski 1981)
Tagus Estuary	Portugal		(Sobral 1985)
Sado Estuary	Portugal		(Monteiro 1995)
Mondego Estuary	Portugal	Western	(Azeiteiro et al. 2000)
	South Pacific		
Port Jackson	Australia		(Dana 1849)
tropical meromictic lake in New Guinea	New Guinea		(De Meester and Vyverman 1997)

Table 1-1, continued: Partial listing of estuaries and coastal regions where *Acartia tonsa* has been described, divided by geographic region.

female production of 46-614 eggs; (Crokett 1967), and has free falling eggs that are easy to collect The populations along the east coast of the USA north of Cape Cod are restricted to estuaries (Paffenhofer and Stearns 1988; Tester and Turner 1991), while those along the West coast of the USA reside in coastal waters. Like most copepods, their development consists of six naupliar stages, five copepodid stages, and an adult stage (Sabatini 1990). Adults are 1 – 1.5mm and they can be sexed during the last

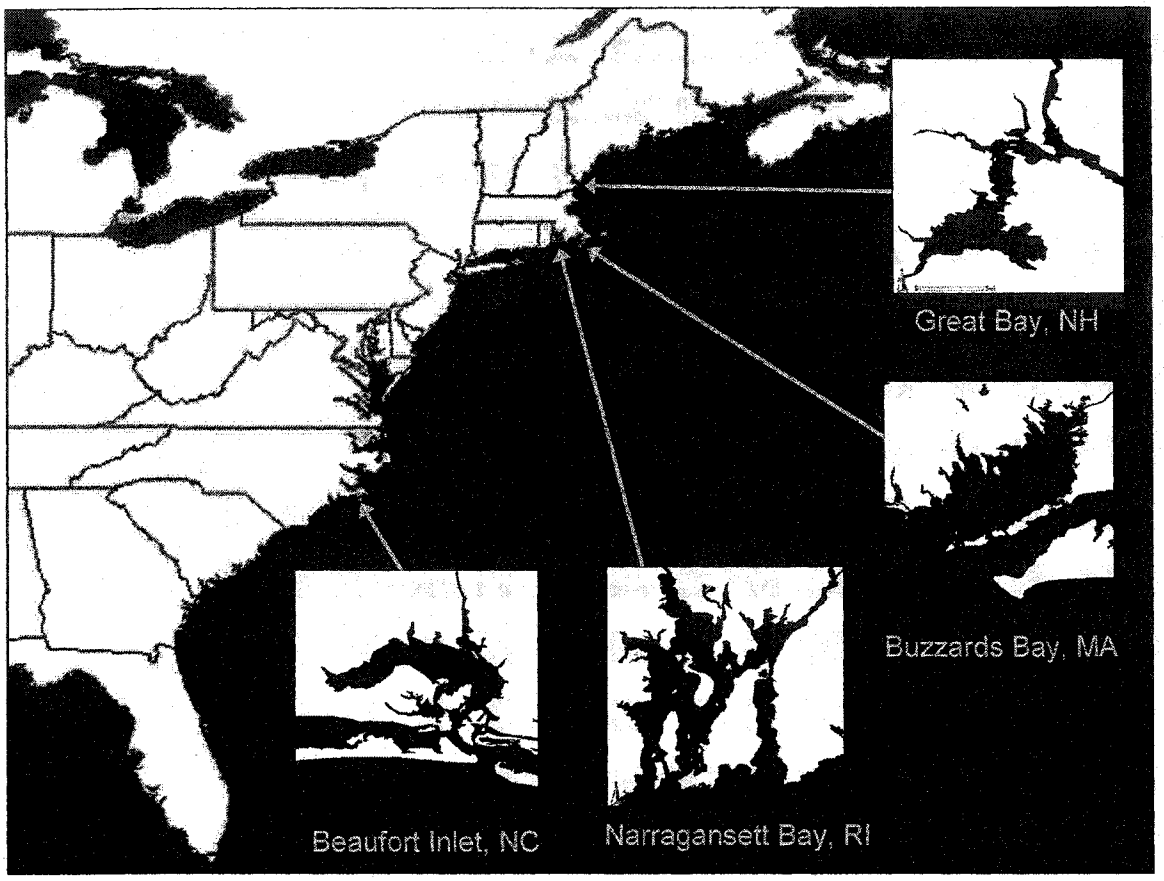

Figure 1-1: Map of the East Coast of the USA showing the sample collection sites: Great Bay, New Hampshire, the dock at the University of New Hampshire's Jackson Laboratory in Durham, NH (43.9075°N; 70.8644°W); Buzzards Bay, Massachusetts, the jetty at Fort Rodman Military Reservation in New Bedford MA (41.5963°N; 70.8995°W); Narragansett Bay, Rhode Island, the pier at the University of Rhode Island's Narragansett Bay campus in Narragansett, RI (41.4916°N; 71.4195°W); and Beaufort Inlet, North Carolina, off the Pivers Island Road Bridge over Gallants Channel in Beaufort, NC (34.7200°N; 76.6731°W).

copepodid stage. Egg production has been widely used as an indicator of fitness to measure the effects of various environmental conditions including: suspended sediment (White and Dagg 1989); exposure to toxins (Johansen and Mohlenberg 1987); food patchiness and perdition (Saiz et al. 1993); seasonal factors (Ambler 1985); and food type and quality (Ambler 1986; Kleppel 1992; Dam et al. 1994; Kleppel and Burkart 1995; Kleppel et al. 1998a; Kleppel et al. 1998b). However, one study found egg hatching success to be a better measure of fitness than egg production (Burkart and Kleppel 1998). *Acartia tonsa* can survive at temperatures up to 42°C and acclimate to temperatures

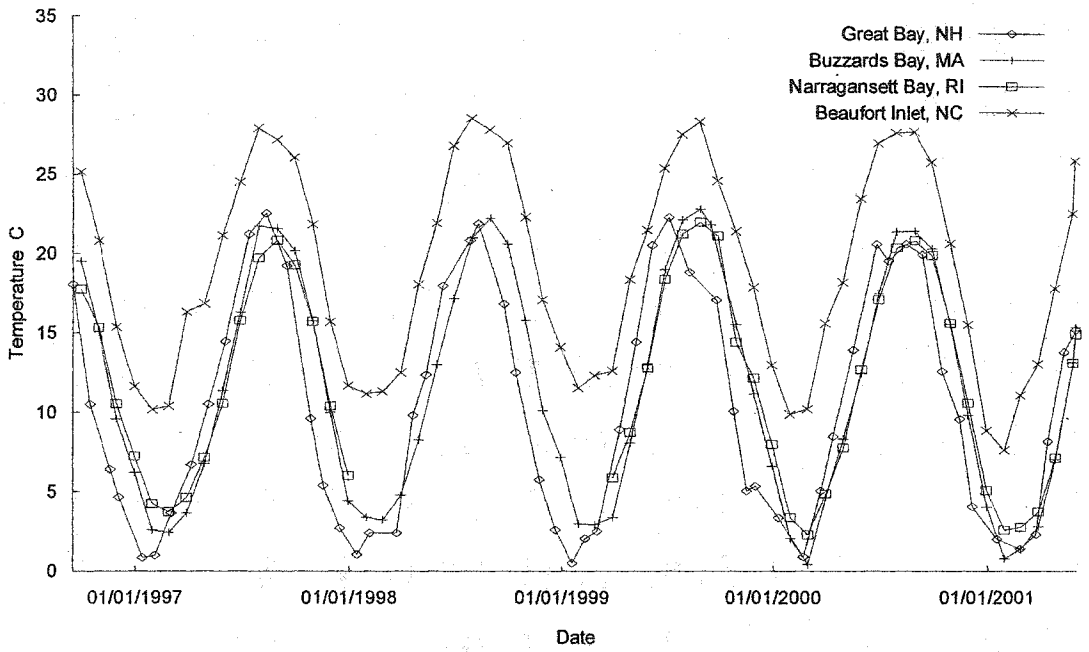

Figure 1-2: Mean monthly temperature from September 1997 to June 2001. Reading taken from Great Bay, NH (at Adam's Point by The Cooperative Institute for Coastal and Estuarine Environmental Technology (CICEET), data available at http://ciceet.unh.edu), Buzzards Bay, MA (at Woods Hole by the Center for Operational Oceanographic Products and Services (CO-OPs), data available at http://co-ops.nos.noaa.gov/), Narragansett Bay, RI (at T-Warf by The National Estuarine Research Reserve System (NERR), data available at http://nerrs.noaa.gov, note that no data was available from January 1998 till March 1999), and Beaufort Inlet, NC (at Beaufort by CO-Ops).

between -1° and 32°C (Gonzalez 1974). Molecularly, McLaren (Dalhousie University, personal communication) has determined the genome size to be between 8.42 x 10^8 and 9.37 x 10^8bp. The number of chromosomes is unknown, but all other *Acartia* species that have been examined have a chromosome numbers of 2N = 12 (female) and 2N = 11 (male) (Goswami and Goswami 1973).

Estuaries are highly variable environments with special and temporal variation in temperature, salinity, nutrients, chemical composition, and other environmental factors. The studies presented here focus on *A. tonsa* from four estuaries along the East Coast of the USA (Figure 1-1): Great Bay, New Hampshire; Buzzards Bay, Massachusetts; Narragansett Bay, Rhode Island; and Beaufort Inlet, North Carolina. One consistent

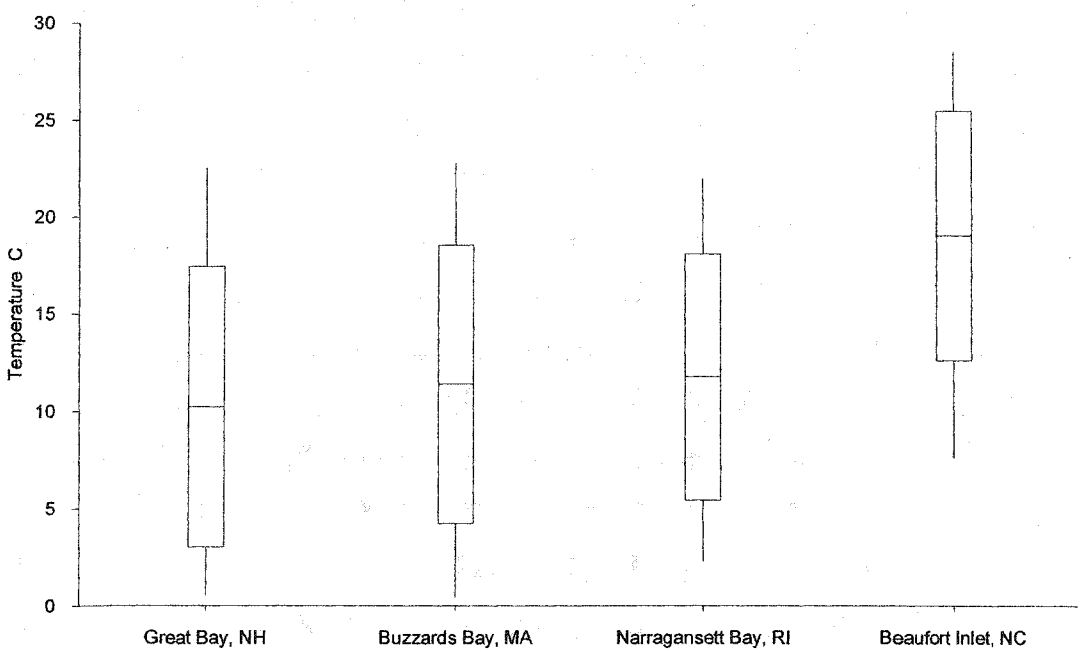

Figure 1-3: Graph showing the mean (middle line), one standard deviation (upper and lower limits of the box) and range (upper and lower limits of line) of temperature from Great Bay, NH, Buzzards Bay, MA, Narragansett Bay, RI, and Beaufort Inlet, NC using same data as Figure 1-2.

variable among the estuaries is temperature, which shows a regular pattern along the east coast of the USA, with ~1°C change in temperature for every 1° change in latitude. This can been seen in monthly temperature records from the estuaries included in this study (Figure 1-2), as well as in the monthly averages over 5 years (Figure 1-3).

A second focus of the study is evolutionary patterns among species of *Calanus*, an important genus of calanoid copepods found in the open ocean. These species also exhibit markedly conserved morphology, with species that are difficult – or in some cases impossible – to distinguish with morphological characters alone. This comparative study demonstrates that the pattern of evolution in calanoid copepods, with significant genetic divergence accompanied by little or no morphological divergence, can occur in diverse taxonomic groups within the order and in diverse marine environments. The life histories

of many *Calanus* species has been describe by Conover (Conover 1988). Detailed studies of the population structure and distribution patterns of *Calanus* in the North Atlantic have been conducted (Daan et al. 2000), many with an emphasis on *Calanus finmarchicus* (Muus 1996).

By examining these estuarine and open ocean populations of copepods, this study seeks to address the question: What is the population structure of calanoid copepods and how much genetic and physiological diversity is contained within this morphologically conserved group? Chapters 2 through 4 of this study examine the differences between populations of *A. tonsa* from the East Coast of the USA. Chapter 2 analyzes the population genetic structure using mitochondrial Cytochrome Oxidase I (mtCOI) and amplified fragment length polymorphisms (AFLP). Chapter 2 also examines the ability of these populations to interbreed with each other. Chapter 3 looks at the physiological response of two of the *A. tonsa* populations to extreme temperatures and chapter 4 examines the types of eggs laid by these populations at low temperature with a short photoperiod. Chapter 5 uses mtCOI sequence data to reconstruct the phylogenetic relationship of 9 *Calanus* species from the open ocean and utilizes the DNA sequence differences to develop a PCR based molecular tool to identify 4 of these species.

CHAPTER II

POPULATION GENETIC STRUCTURE AND PHYLOGENETIC INFERENCES OF *ACARTIA TONSA* FROM FOUR ESTUARIES

ABSTRACT

The population genetic structure and interbreeding success of the estuarine copepod *Acartia tonsa* collected from four estuaries along the East Coast of the USA were analyzed. The copepods were collected from Great Bay, New Hampshire (NH); Buzzards Bay, Massachusetts (MA); Narragansett Bay, Rhode Island (RI); and Beaufort Inlet, North Carolina (NC). The population genetic structure was analyzed using DNA sequence from a 713bp fragment of the mitochondrial Cytochrome Oxidase I (mtCOI) gene and Amplified Fragment Length Polymorphism (AFLP) fragments from two primer pairs. An Analysis of Molecular Variance (AMOVA) of the mtCOI sequence data identified significant genetic structure between three groups: NH; MA and RI; and NC. A Bayesian population genetic analysis of the AFLP data showed significant genetic structure between the MA and RI populations. However, the mean genetic difference between the populations suggested there is only a very small restriction on the exchange of genetic material between the estuaries. To test the ability of individuals from the four collection sites to breed with each other, all combinations of reciprocal crosses were established. All crosses produced F_1 and F_2 generations except when NH males or females were crossed with any other collection site. The F_1 progeny of NH crossed with

MA, RI, or NC were all arrested in the third naupliar stage of development. These findings indicate that *A. tonsa* from NH is a different species than *A. tonsa* from MA, RI, and NC based on biological and evolutionary species concepts. Further, this study suggests that estuarine copepods (and probably other obligate estuarine organisms) harbor a large amount of genetic diversity, and that currently identified species may include cryptic species.

INTRODUCTION

The genetic diversity of copepods has traditionally been underestimated, possibly due to the morphological similarities that exist even between distantly related species (Bucklin et al. 2001; Hill et al. 2001; Rocha-Olivares et al. 2001). Many estuarine copepods have been described as having extensive geographic ranges (i.e. *A. tonsa*, *A. clause*, *A. longiremis*, *Eurytemora affinis*, and others). However, growing evidence suggests that such species may not constitute a single evolutionary group, or even a single species.

Several studies have reported reproductive isolation between estuarine populations of benthic and meiobenthic harpacticoid copepods. Burton and others (Burton and Place 1986; Burton 1990b; Burton 1990a; Ganz and Burton 1995; Rawson and Burton 2002) crossed populations of *Tigriopus californicus* from several estuaries along the coast of California and Baja California and found incompatibilities ranging from reduced fitness in the F_2 generations to failure to produce viable eggs. A population of *Scottolana canadensis* from the coast of Florida was shown to be reproductively isolated from

populations along the East Coast of the USA (Maine to South Carolina; (Lonsdale et al. 1998). *Cletocamptus deitersi* populations from estuaries in Louisiana, Alabama, and California were shown to have large genetic divergences when analyzed with mtCOI and mt16S rDNA sequence data and are suggested to represent subspecies (though no breeding experiments were done; (Rocha-Olivares et al. 2001). Genetic studies on another harpacticoid copepod, *Microarthridion littorale*, revealed significant structure between geographically close populations from estuaries off the coast of North Carolina and Georgia (Schizas et al. 2002).

Fewer studies have examined population genetics of estuarine species of planktonic calanoid copepods. One study showed that populations of *Acartia clausi* from the East and West Coast of the United States were reproductively isolated from each other (Carrillo et al. 1974). A study of two populations of *Acartia californiensis* (from California and Baja California) showed no reduction in fitness in the hybrid progeny (Trujillo-Ortiz 1990). Another study examined the population structure of *A. tonsa* using mt16s rDNA sequence data from estuaries along the East and West Coasts of the United States and the Gulf of Mexico. The study revealed strong population genetic structure indicating little migration between estuaries (Caudill and Bucklin 2004). Lee (1999) used mtCOI sequence evidence to show multiple freshwater invasions of the estuarine and freshwater calanoid copepod *Eurytemora affinis*.

The objective of this study was to determine the population genetic structure, genetic diversity, and interbreeding ability of populations of *A. tonsa* from the East Coast of the USA. To accomplish this, *A. tonsa* were collected from four different geographic

locations along the East Coast of the USA (Great Bay, NH, Buzzards Bay, MA, Narragansett Bay, RI, and Beaufort Inlet, NC). These estuaries differ with regard to mean annual temperature, tidal influence, predators, and chemical composition. Population genetic diversity and structure were examined with two kinds of genetic markers, mtCOI DNA sequences and amplified fragment length polymorphisms (AFLP). The two types of genetic markers were chosen for their levels of variability. mtCOI DNA sequence is a highly variable protein coding sequence of known function with strong functional constraints at replacement sites based on analytical data (Brown et al. 1976; Brown et al. 1982). AFLPs are extremely variable markers representing changes in the nuclear genome whose functions are unknown. While the evolutionary pattern of any one AFLP fragment is unknown, it is assumed that on the whole they are not under positive selection (Vos et al. 1995).

MATERIALS AND METHODS

Field collections and laboratory culture:

Acartia tonsa adults were collected during the same time period from four locations: the dock at the University of New Hampshire's Jackson Laboratory in Durham New Hampshire on Great Bay (43.9075°N; 70.8644°W) on October 31, 2000; the jetty at Fort Rodman Military Reservation in New Bedford Massachusetts on Buzzards Bay (41.5963°N; 70.8995°W) on November 2, 2000; the pier at the University of Rhode Island's Narragansett Bay campus in Narragansett Rhode Island on Narragansett Bay

(41.4916°N; 71.4195°W) on November 2, 2000; and off the Pivers Island Road bridge over Gallants Channel in Beaufort Inlet near Beaufort North Carolina (34.7200°N; 76.6731°W) on October 28, 2000. All samples except NC were collected by R. S. Hill using a 333μm mesh net with a quart glass jar as a cod end. Samples were collected at high tide by tossing the net into the water, letting it sink below the surface, and then slowly pulling it up and through the water column. The samples were filtered through a 2000μm mesh into a clean glass jar and stored in a cooler with ice packs for transportation to the laboratory where *A. tonsa* were identified and removed to a separate container. Identification of *A. tonsa* in all cases was based on the morphology of the fifth thoracic leg of adult females which is distinct from other *Acartia* that are found in these estuaries, only females were sorted from the collected samples. For the NC samples, net tows were made off Pivers Island road bridge and individual *A. tonsa* were identified by Patricia Tester (National Oceanic and Atmospheric Administration, Southeast Fisheries Science Center), fed, and placed in a one-quart cooler for overnight shipment to UNH. In the laboratory, 20 – 30 fertilized adult females were placed in 2L glass bottles with three 2L cultures started for each collection site. The bottles were filled with water collected with the samples and filtered through a 20μm mesh. Cultures were grown at room temperature with a 14:10 hr light:dark cycle. Every other day 200ml of water was removed from each culture using a siphon fitted with a 20μm mesh and 200ml of 20ppt Instant Ocean was added with a mixture of the phytoplankton *Rhodomonas salina* (CCMP1319) and *Isochrysis galbana* (CCMP1323). The phytoplankton were grown in Guillard's f/2-si media in 33ppt Instant Ocean at room temperature with a 14:10 hr light:dark cycle.

Sequencing of COI:

A 713bp fragment of mitochondrial COI (mtCOI) was sequenced from *Acartia tonsa* individuals from the four collection sites. Genomic DNA was extracted from groups of 50 adult individuals from each collection site using a phenol:chloroform protocol. The mtCOI gene was amplified using the universal PCR primers COI-1490 and COI-2198 (Folmer et al. 1994). The PCR products were cloned into a pCR-4-TOPO plasmid and transformed into TOP10 Chemically Competent cells using Invitrogen's TOPO TA Cloning Kit for Sequencing following the recommended protocol. Cells were grown and plated, and individual colonies were checked for the mtCOI insert by PCR amplification with the COI-1490 and COI-2198 primers (Folmer et al. 1994). Plasmid DNA was isolated from 30 insert-positive colonies for each collection site using Promega's Wizard Mini Prep System as prescribed. The 96 plasmids were cycle sequenced in both directions using Invitrogen's T3 and T7 sequencing primers with Amersham's Dyenamic sequencing reaction mix and run on an ABI 377 sequencer with 96 lanes. The sequence data were analyzed and aligned using ProSeq (Filatov 2001). Of the 30 plasmids sequenced from each collection site, high quality reads were obtained from 25 NH, 26 MA, 23 RI, and 24 NC plasmids. Molecular distance, parsimony calculations, and tree building were all done using MEGA2 (Kumar et al. 2001). Distance trees were calculated using the Tamura-Nei gamma method (Tamura and Nei 1993) and trees were constructed using the neighbor-joining method (Saitou and Nei 1987) and bootstrap values (Felsenstein 1985). The alpha parameter of the gamma shape distribution used for molecular distance calculations was determined using the baseml program in Paml (Yang

2002). Analysis of Molecular Variance (AMOVA) was done using Arlequin (Schneider et al. 2000).

AFLP analysis:

Genomic DNA was extracted from individual adult *A. tonsa* using a guanidinium thiocyanate protocol which comprised the following steps: 1. live individuals were placed in a 1.5µl micro-centrifuge tube and excess water was removed; 2. 50µl of buffer (4M guanidinium thiocyanate, 25mM Na citrate pH 7.0, 0.5% SDS, 0.1M β-mercaptoethanol, and 0.1mM dithiothreitol) was added and samples were thoroughly ground against the side of the tube using a micro-pestle; 3. the solution was incubated at 65°C for 10min mixing every 2min; 4. the solution was extracted with 50µl phenol; chloroform; isoamyl alcohol (25:24:1) and the aqueous layer transferred to a fresh tube; 5 50µl of dH2O was added to the organic layer, re-mixed and separated, and the aqueous layer pulled off and added to the previous aqueous layer; 6. the mixed aqueous layers were extracted in an identical manner with 100µl chloroform; isoamyl alcohol (24:1); 7. 3µl of 5mg/ml RNase A was added and incubated for 2hr at 37°C; 8. DNA was precipitated with 15µl Na acetate pH 5.2 and 300µl ice cold ethanol; 9. the sample was incubated for at least 1hr at -20°C; 10. the sample was centrifuged for 1hr at 0°C; 11. ethanol was pulled off and pellet washed with 400 µl ice cold 70% ethanol; 12. the sample was centrifuged for 2min at 0°C; 13. ethanol was pulled off and pellet allowed to dry on bench top; and 14. the pellet was resuspended dH2O. The entire DNA extraction was used in the AFLP procedure as described by Vos et al (Vos et al. 1995). Two sets of primer pairs were used in this study: MSE-CAC with EcoRI-ACT (Fam) and MSE-CAG

with EcoRI-AGC (TAMARA), these two primer sets were determine to produce the highest yield of bands out of 6 primer pairs tested. Twenty-four samples from each collection site were run multiplexed for the two sets of primer on an ABI-377 sequencer with 96 lanes. Only lanes with strong signal for both dyes and a clean read of the internal size standard were used. This left 18 NH, 15 MA, 18 RI, and 13 NC samples. Fragments were identified on the gel image and manually categorized into bins using the program Genographer (Benham 2001), only fragments between the sizes of 75bp and 490bp were used. Genetic distance and tree reconstruction were analyzed with MEGA, and estimates of heterozygosity and Θ^B (F_{ST}) were calculated with a Bayesian population genetic analysis using Hickory (Holsinger and Lewis 2003).

Interbreeding experiments:

To ensure that all experimental copepods were virgins, individuals in stages NV-CIV were put in 60ml glass jars containing 25ml 20ppt Instant Ocean and grown to maturity in isolation (but otherwise in standard culture conditions). Once the copepods matured, they were checked for vitality and sexed. Crosses were established by gently pouring the copepod from one bottle into a bottle containing another copepod. Crosses were established between individuals from the same collection site and between individuals from different collection sites in a reciprocal manner with all possible combination of crosses: NH♀xNH♂, NH♀xMA♂, NH♀xRI♂, NH♀xNC♂, MA♀xNH♂, MA♀xMA♂, MA♀xRI♂, MA♀xNC♂, RI♀xNH♂, RI♀xMA♂, RI♀xRI♂, RI♀xNC♂, NC♀xNH♂, NC♀xMA♂, NC♀xRI♂, and NC♀xNC♂. Twenty-four of each combination of crosses were established. Each cross was checked every 7 days for a period of 42 days. Once

	NH	MA	RI	NC
NH	0.011			
MA	0.155	0.009		
RI	0.154	0.008	0.006	
NC	0.156	0.014	0.016	0.003

Table 2-1: Mean genetic distance between and within *A. tonsa* from different collection sites based on sequence data from a 713bp fragment of mtCOI calculated using the Tamura-Nei equation ($\alpha = 0.83$).

nauplii appeared in a bottle, the original parents were removed. If F_1 nauplii appeared, they were allowed to develop and generate an F_2 generation if they could. For each cross, it was recorded if eggs were laid, if the eggs hatched into nauplii, if the nauplii matured, if the F_1 adults could produce F_2 nauplii, and if the F_2 nauplii matured.

The mating results were analyzed as contingency tables using a G-test of independence with either a Williams's or Yate's correction. Subsequent unplanned tests of homogeneity were done where applicable using the G-test (Sokal and Rohlf 1995).

RESULTS

mtCOI sequence variation:

The mtCOI sequencing resulted in 19 NH haplotypes, 19 MA haplotypes, 16 RI haplotypes, and 16 NC haplotypes. Of these, all of the NH and NC haplotypes were unique to their respective populations, and MA and RI shared 3 haplotypes. The mean genetic distances calculated using the Tamura-Nei equation with a gamma distribution alpha value of 0.83 (Table 2-1) for within collection sites were NH = 0.011, MA = 0.009, RI = 0.006, and NC = 0.003. The mean genetic distances between collection sites were NH and MA = 0.155, NH and RI = 0.154, NH and NC = 0.156, MA and RI = 0.008, MA

	NH	MA	RI	NC
NH	0.007			
MA	0.007	0.005		
RI	0.006	0.004	0.003	
NC	0.007	0.005	0.004	0.004

Table 2-2: Mean poisson corrected genetic distance between *A. tonsa* from different collection sites based on amino acid sequence data from a 237aa fragment of mtCOI.

and NC = 0.014, and RI and NC = 0.016. A neighbor joining tree including all collection sites was generated using Tamaura-Nei distances ($\alpha = 0.83$; Figure 2-1). The same tree was bootstrapped 1000 times and condensed to only show branches supported by greater than 70% bootstrap values (Figure 2-2, left) and a consensus maximum parsimony tree of the same sequence data showing only branches supported by greater than 70% of the trees was generated (Figure 2-2, right). The Tamura-Nei neighbor-joining tree and the maximum parsimony tree are very similar. Both trees show lineage separation of the NH samples. Both trees also clustered all of the NC samples. The MA and RI samples interspersed with each other. Support for selective constraint on the mtCOI gene between these populations is seen in trees generated using the 237 amino acid translation of the DNA sequence data (Figure 2-3). NH had 13 aa sequence haplotypes, MA had 10, RI had 7, and NC had 10. There is one haplotype that is shared among all collection sites, while all of the others are unique to their collection site. There are no amino acid changes conserved within one collection site and not present in the others. The mean genetic differences based on a Poisson corrected calculations of the amino acid sequences were NH = 0.007, MA = 0.005, RI = 0.003, NC = 0.004, NH and MA = 0.007, NH and RI = 0.006, NH and NC = 0.007, MA and RI = 0.004, MA and NC = 0.005, and RI and NC = 0.004 (Table 2-2). The amino acid analysis shows a cluster of some but not all of the NH sequences and no structure among the remaining samples. This shows that there was a high synonymous to non-synonymous mutation ratio and implies that there has

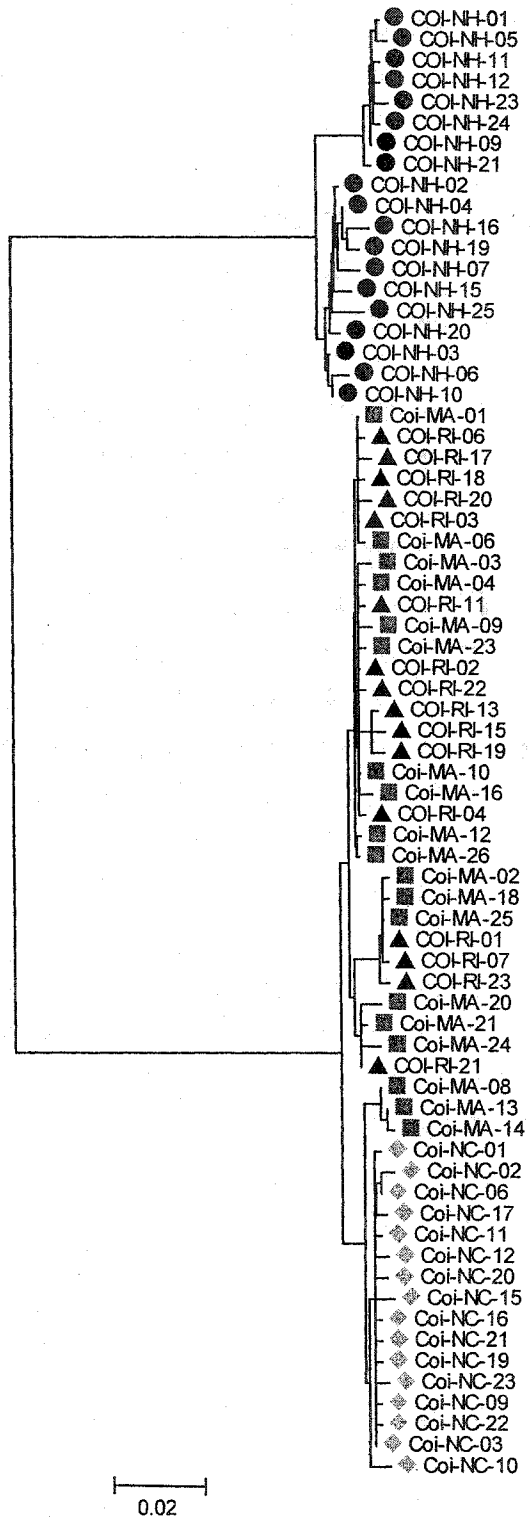

Figure 2-1: Tamura-Nei (α = 0.83) neighbor joining tree of *A. tonsa* based on a 713bp fragment of DNA sequence of mtCOI. Circles = NH; Triangles = RI; Squares = MA; and Diamonds = NC, Bar = Distance.

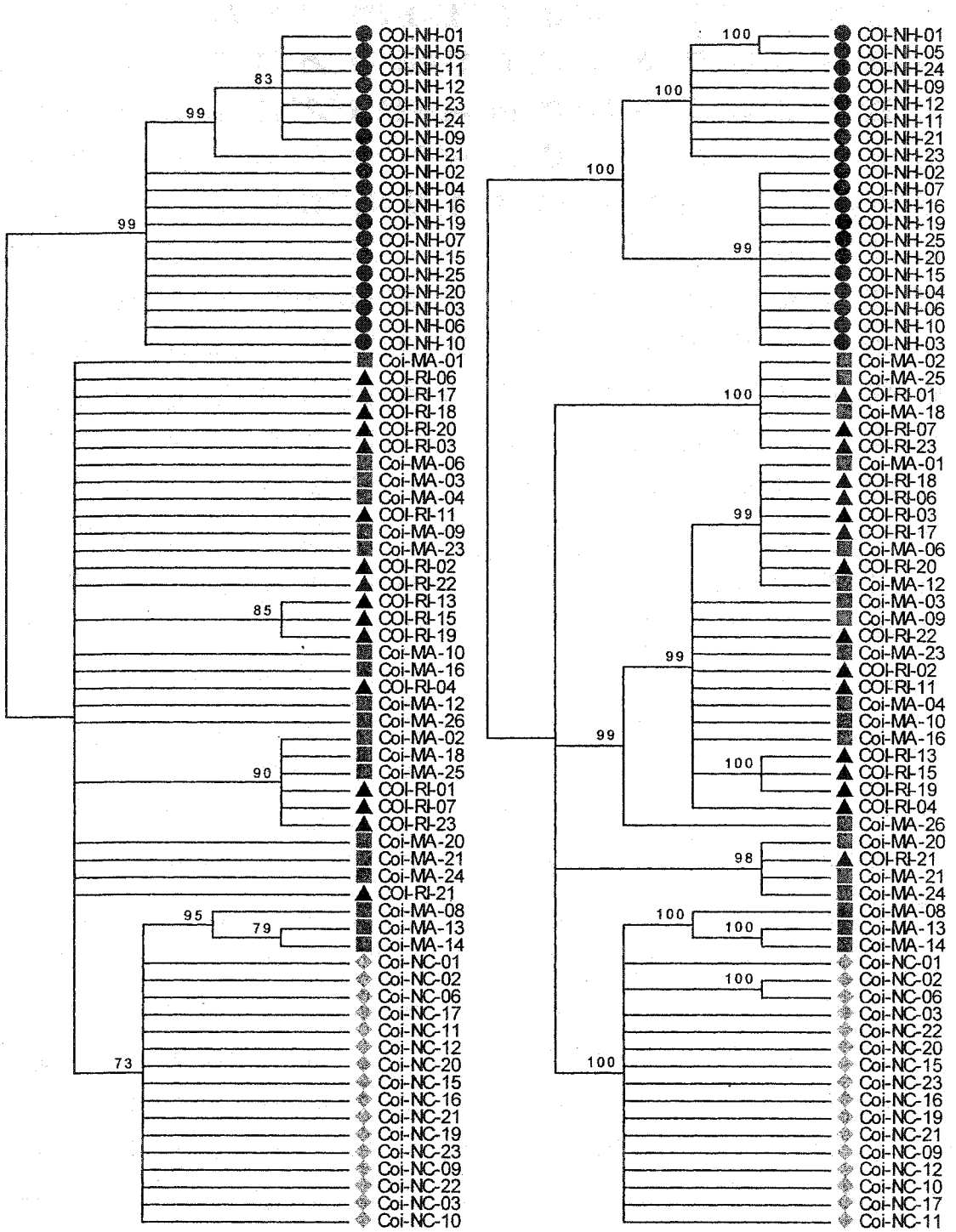

Figure 2-2: Two trees of *A. tonsa* from different collection sites based on sequence data from a 713bp fragment of mtCOI. Left: Tamura-Nei (α=0.83) neighbor joining tree, bootstrapped 1000X, condenced to show only branches supported by >70% bootstrap values. Right: Consensus maximum parsimony tree showing only branches supported by >70% of the trees. Circles = NH; Triangles = RI; Squares = MA; and Dimonds = NC; Numbers = bootstrap values or percent of supporting trees.

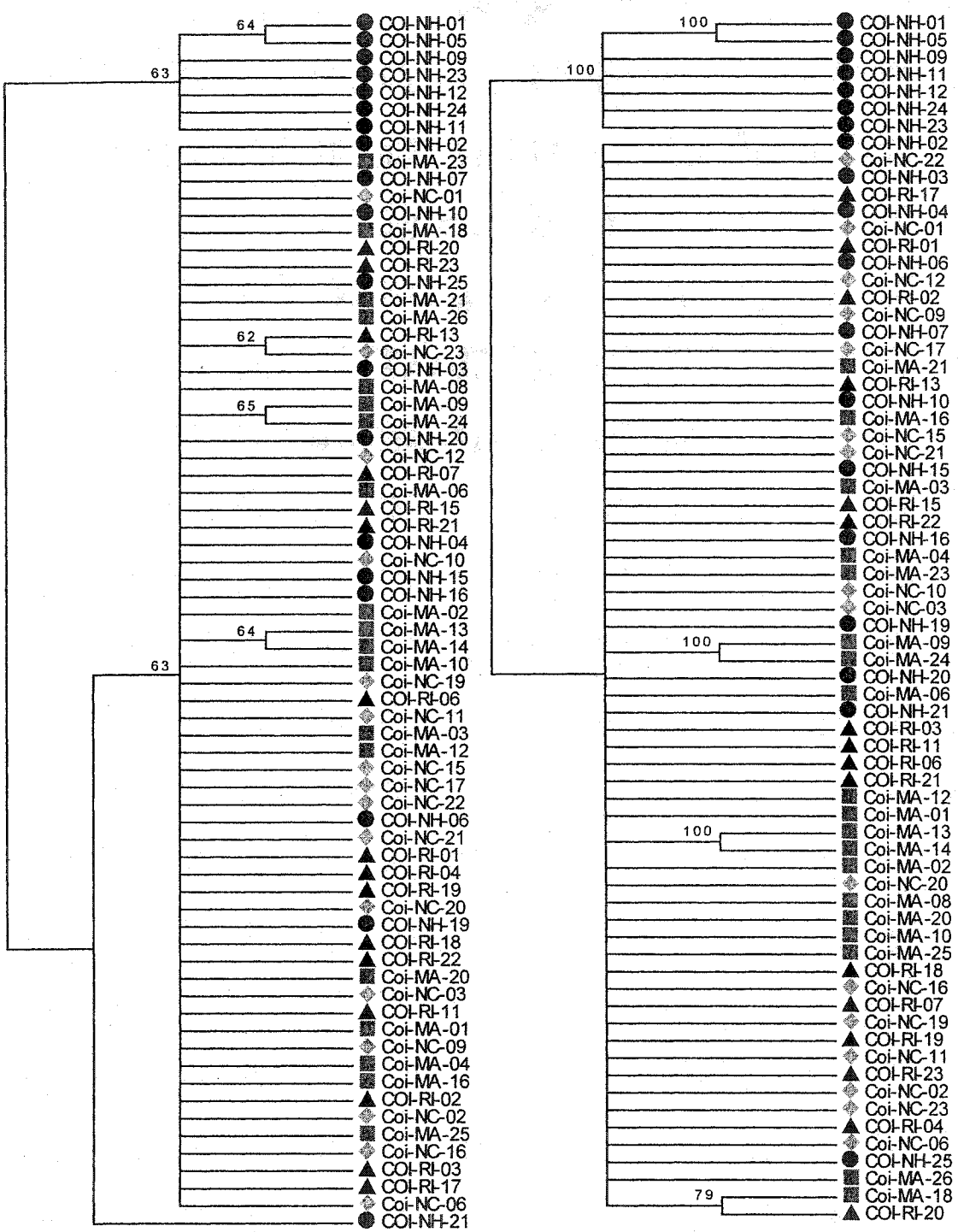

Figure 2-3: Two *A. tonsa* trees based on 237 amino acid fragment of mtCOI. Left: Poisson corrected neighbor joining tree, bootstrapped 1000X, condensed to show only branches supported by >50% bootstrap values. Right: Consensus maximum parsimony tree showing only branches supported by >50% of the trees. Circles = NH; Triangles = RI; Squares = MA; and Diamonds = NC, Numbers = bootstrap values.

Comparison	Φ_{ST}	p-value
All Locations	0.90	<0.0001
NH vs. MA	0.92	<0.0001
NH vs. RI	0.93	<0.0001
NH vs. NC	0.94	<0.0001
MA vs. RI	0.03	0.067
MA vs. NC	0.57	<0.0001
RI vs. NC	0.70	<0.0001

Table 2-3: AMOVA results based on comparison of a 713bp fragment of DNA sequence of mtCOI between *A. tonsa* individuals from different collection sites.

been selection against functional changes in the amino acid sequence.

An analysis of molecular variance (AMOVA) was performed on the mtCOI DNA sequence data between all of the *A. tonsa* collections. All tests of significance were based on 100,000 permutations. A significant ($p < 0.0001$) Φ_{ST} of 0.90 was calculated for all collection sites together (Table 2-3). Significant ($p < 0.0001$) Φ_{ST} were also found for all but one pairwise collection site comparisons as follows: NH and MA = 0.92; NH and RI = 0.93; NH and NC = 0.94; MA and NC = 0.57; and RI and NC = 0.70 (Table 2-3). MA and RI generated an insignificant ($p = 0.067$) Φ_{ST} of 0.03 (Table 2-3). These results corroborate the structure identified in the phylogenetic tree. There is little to no migration between the NH estuary and the other estuaries. There is also highly restricted gene flow between the NC estuary and the RI and MA estuaries.

AFLP variation:

AFLP markers are more variable than mtCOI gene sequences and are therefore more informative when analyzing closely related populations. The two AFLP primer pairs used in this study produced 110 fragments, 92 of which were polymorphic among the four *A. tonsa* populations looked at here. The NH individuals contained 90 polymorphic

bands (mean heterozygosity = 0.42±0.01), the MA individuals contained 88 polymorphic bands (mean heterozygosity = 0.41±0.01), the RI individuals contained 91 polymorphic bands (mean heterozygosity = 0.42±0.01), and the NC individuals contained 89 polymorphic bands (mean heterozygosity = 0.41±0.01). Each individual had a unique genotype. The mean proportion of pairwise differences was 0.364 within NH, 0.343 within MA, 0.366 within RI, 0.336 within NC, 0.379 between NH and MA, 0.383 between NH and RI, 0.366 between NH and NC, 0.380 between MA and RI, 0.356 between MA and NC, and 0.362 between RI and NC. The levels of variation observed with the mtCOI sequence data suggested that the highly polymorphic AFLP data was probably saturated, at least for the NH population compared to the other populations, therefore, further analysis was only conducted on the MA, RI, and NC individuals. Neighbor joining trees based on the proportion of pairwise differences between MA, RI, and NC individuals (Figure 2-4, left) and between individuals of the two closest populations, MA and RI (Figure 2-4, right), were generated. The lack of grouping of the NC individuals in the tree indicates that the AFLP data is perhaps saturated at the distance between the MA and RI populations and the NC population. The MA and RI tree shows some weak clustering of individuals from the same collection site. To determine if there was a significant population structure between these geographically close populations, the mean Θ^B value (similar to F_{ST}).was calculated using AFLP data for MA and RI individuals (mean Θ^B = 0.042 with a 95% confident interval of 0.024 – 0.065). While the Θ^B is significant (the lower 95% limit is greater than 0), it is so low that the differences between the populations may not be that meaningful.

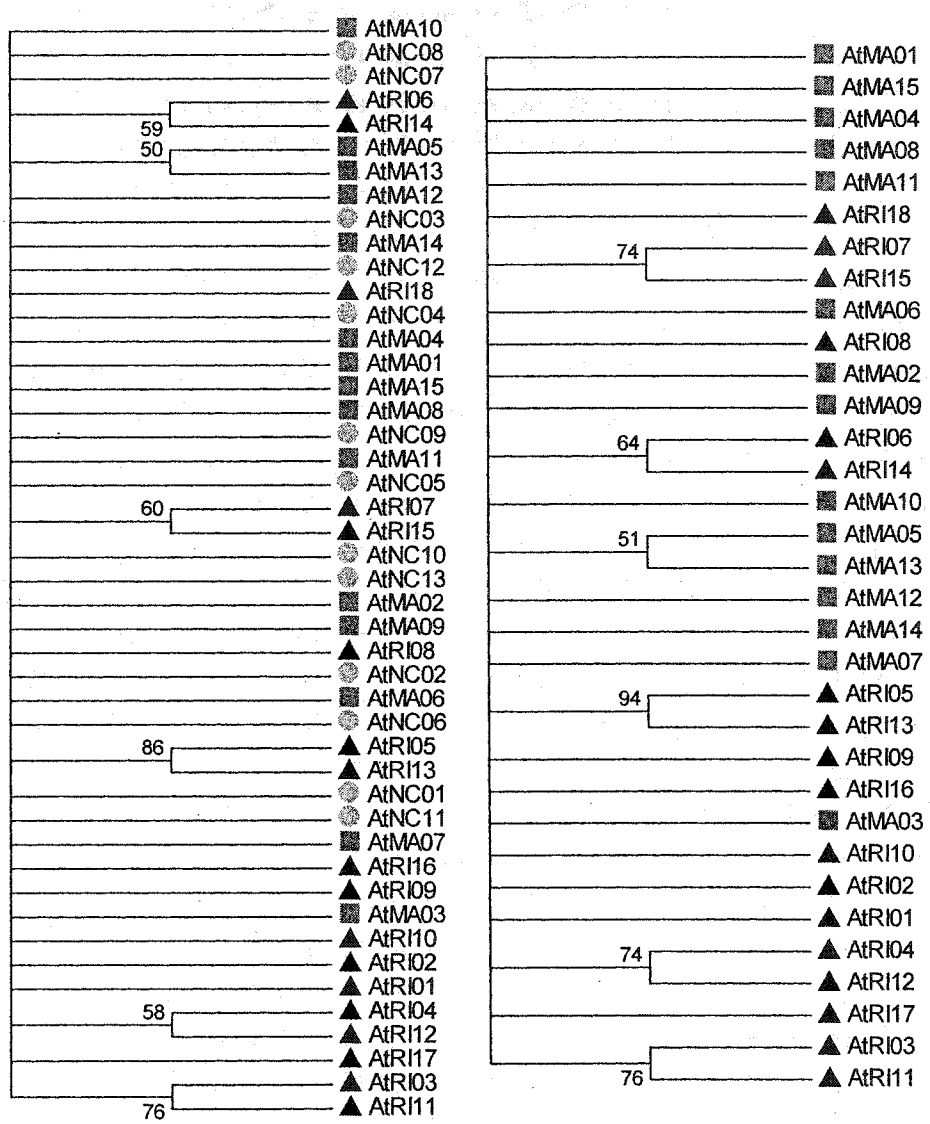

Figure 2-4: ***Acartia tonsa*** **neighbor Joining trees based on mean proportion of pairwise differences of AFLP markers, bootstrapped 1000X, condenced to show only branches supported by >50% bootstrap values. Left: MA (squares), RI (Triangles), and NC (circles). Right: MA and RI.**

	NH♀			MA♀			RI♀			NC♀		
	F	N	F_2	F	N	F_2	F	N	F_2	F	N	F_2
NH♂	6 (25)	18 (75)	18 (75)	16 (67)	8 (33)	0 (0)	14 (58)	10 (42)	0 (0)	15 (63)	9 (38)	0 (0)
MA♂	16 (67)	8 (33)	0 (0)	9 (38)	0 (0)	15 (63)	12 (50)	0 (0)	12 (50)	14 (58)	0 (0)	10 (42)
RI♂	13 (54)	11 (45)	0 (0)	7 (29)	0 (0)	17 (70)	8 (33)	0 (0)	16 (67)	12 (50)	0 (0)	12 (50)
NC♂	14 (58)	10 (41)	0 (0)	12 (50)	0 (0)	12 (50)	9 (38)	0 (0)	15 (63)	11 (46)	0 (0)	13 (54)

Table 2-4: Results of crosses: number and (percentages). F = number of crosses that failed to produce nauplii, N = number of crosses that produced nauplii that failed to develop beyond the third naupliar stage, and F_2 = number of crosses that were able to produce an F_2 generation.

Interbreeding:

The results of 384 matings fell into three classes: (1) produced eggs that were not fertilized or failed to hatch; (2) eggs hatched but only developed to naupliar stage III; and (3) eggs hatched and proceeded through an F_2 generation (Table 2-4). Between 25% and 67% of matings in every group of crosses failed to produce nauplii (Table 2-5), in most cases however, males did attach spermatophores to the females. A G-test of independence with a Williams's correction (to account for the small number of individuals in each category) comparing the number of failed to non-failed matings in each group of crosses showed no significant difference between groups ($p = 0.0513$). While this probability is greater than the significant cutoff of $p < 0.05$ set *a priori*, it is worth noting that crosses between individuals from different collection sites tended to have more failed matings than crosses between individuals from the same collection site (the exception being NC X NC cross) and the most failed matings occurred in crosses involving NH individuals and a non-NH individual (the exception being NC X MA cross). The second class, eggs hatched but juveniles failed to develop, was exclusive to crosses between NH individuals and non-NH individuals. In all of these cases, the

Group	Failed	Not-Failed
NHXNH	6	18
NHXMA	16	8
NHXRI	13	11
NHXNC	14	10
MAXNH	16	8
MAXMA	9	15
MAXRI	7	17
MAXNC	12	12
RIXNH	14	10
RIXMA	12	12
RIXRI	8	16
RIXNC	9	15
NCXNH	15	9
NCXMA	14	10
NCXRI	12	12
NCXNC	11	13

Table 2-5: Contingency table of the number of matings that Failed to produce any nauplii verses those that produced nauplii (Not-Failed) for each cross. G-test of independence shows no significant difference between groups (p=0.0513).

nauplii developed normally to the stage III nauplius then failed to molt to the stage IV nauplius, surviving for an extended period as stage III nauplius. The third class, eggs hatched and proceeded through an F_2 generation, occurred in all crosses not involving a NH and non-NH individual. A G-test of independence with Yate's correction (to account for the zeros in the data) comparing the number of crosses that produced only nauplii to the number of crosses that produced an F_2 generation for each group, showed a highly significant difference between groups ($p < 0.0001$; Table 2-6). A G-test of homogeneity reveals that the crosses involving NH individuals mated to non-NH individuals were not significantly different from each other ($p = 1.00$) and the remaining crosses were not significantly different from each other ($p = 1.00$; Table 2-7).

Group	Nauplii Only	F_2
NHXNH	0	18
NHXMA	8	0
NHXRI	11	0
NHXNC	10	0
MAXNH	8	0
MAXMA	0	15
MAXRI	0	17
MAXNC	0	12
RIXNH	10	0
RIXMA	0	12
RIXRI	0	16
RIXNC	0	15
NCXNH	9	0
NCXMA	0	10
NCXRI	0	12
NCXNC	0	13

Table 2-6: Contingency table of the number of matings that produced nauplii that arrested development at N2 (Nauplii Only) verses the matings that produced nauplii that developed to an F_2 generation. A G-test of independence (Yate's corrected) showed highly significant difference between groups ($p<0.0001$).

	Set 1	
Group	Nauplii Only	F_2
NHXNH	0	18
MAXMA	0	15
MAXRI	0	17
MAXNC	0	12
RIXMA	0	12
RIXRI	0	16
RIXNC	0	15
NCXMA	0	10
NCXRI	0	12
NCXNC	0	13

	Set 2	
Group	Nauplii Only	F_2
NHXMA	8	0
MAXNH	8	0
NCXNH	9	0
NHXNC	10	0
RIXNH	10	0
NHXRI	11	0

Table 2-7: Homogeneous sets within the Nauplii Only verses F_2 data identified by a G-test as having the larges sets with $p>0.05$; for both sets $p=1.00$.

DISCUSSION

Population genetic diversity of *Acartia tonsa:*

At least two cryptic species are likely to exist within the currently described *Acartia tonsa*. One is found in Great Bay, NH, and the other includes populations from Buzzards Bay MA, Narragansett Bay RI and Beaufort Inlet NC. Within this second putative species, significant population genetic differences were observed between MA/RI and NC, suggesting further evidence of possible genetic divergence.

These findings are in good agreement with those presented by Caudill and Bucklin (2004), who examined mt16s rDNA sequence variation of *A. tonsa* from four Atlantic coast estuaries (Great Bay, NH; Buzzards Bay, MA; Narragansett Bay, RI; and Savannah River Estuary, GA), a Gulf of Mexico estuary (Nueces Bay, TX), and a Pacific Coast estuary (LaJolla, CA). In addition to finding strong genetic structure among the Atlantic populations, they found enough genetic differentiation between the Pacific and Atlantic populations to declare it highly likely that they were different species and enough genetic difference between the Atlantic and Gulf of Mexico species to suggest that they too may be different species. The amount of genetic variation they reported between the Pacific, Gulf of Mexico, and Atlantic populations was much greater than the amount of genetic variation they reported among the Atlantic populations. Combining these results with those of the current study which showed that the more closely related populations along the Atlantic cost are different species, strongly implies that the Gulf of Mexico and Pacific Coast population are different species under both the phylogenetic and evolutionary species concepts (see Mayden 1997).

The results of this study indicate that the current concept of a world wide distribution of *A. tonsa* as a single species must be revisited. Further genetic and mating experiments on *A. tonsa* will likely unravel a variable number of cryptic species depending on the definition of species used by different researchers. More important than renaming the different emergent species are the insights into of the evolutionary relationships among the populations. Many questions remain unanswered: Is the observed genetic diversity due strictly to genetic drift? Is the breeding barrier a result of drift or selection? Do the highly variable environmental conditions of estuaries promote genetic diversity or differentiation? In order to understand the evolution of the group, it is suggested that future studies clearly append the name *A. tonsa* with the location of where the population came from (e.g. *A. tonsa*-Great Bay NH USA).

Population genetic structure of estuarine copepods:

Acartia tonsa is not the only copepod that is likely to comprise cryptic species. Indeed, copepod species have traditionally been difficult to distinguish, even those separated by millions of years of evolution (Bucklin et al. 2001; Hill et al. 2001). It might be that there are selective constraints on the basic body type of copepods that has resulted in the morphological similarity among genetically distinct species. Cryptic species of copepods even exist in the open ocean among populations not physically separated (Bucklin et al. 2001). It is thus easy to envision rapid genetic differentiation occurring among isolated populations of copepods that usually exhibit large population sizes and short generation cycles.

Many studies that have examined the genetic relationship of copepod species between estuaries have found genetic structure or inbreeding difficulties. Reduced or restricted breeding was found in *Tigriopus californicus* from the coast of California and Baja California (Burton and Place 1986; Burton 1990b; Burton 1990a; Ganz and Burton 1995; Rawson and Burton 2002), *Acartia clausi* from the East and West Coast of the United States (Carrillo et al. 1974), and *Scottolana canadensis* from along the East Coast of the United States (Lonsdale et al. 1998). Strong population genetic structure was observed in *Eurytemora affinis* through multiple invasions of freshwater from estuaries (Lee 1999), in *Cletocamptus deitersi* populations from estuaries in Louisiana, Alabama, and California (Rocha-Olivares et al. 2001), and *Microarthridion littorale* off the cost of North Carolina and Georgia (Schizas et al. 2002). Taken together, these studies suggest that estuarine copepods are more genetically diverse than previously thought, and that this might be the rule rather than the exception.

Dispersal patterns and mechanism of estuarine copepods:

The genetic diversity among copepods from different estuaries can indicate the mechanisms for dispersal of the organism. Caudill and Bucklin (2004) suggested from their data that the genetic structure of *A. tonsa* is consistent with a northern expansion of *A. tonsa* after the last glacier retreated from the northeastern USA. The results of this study agree with this theory. Among the four estuaries in the study, Great Bay NH is the most isolated, receiving ocean water that originates from the Artic Ocean. Buzzards Bay and Narragansett Bay are in close proximity to each other and intermixing of the populations must occur. The small amount of mixing between the NC estuary and the

MA and RI estuaries could be explained in several ways: estuary-to-estuary transport up the coast; residual genetic similarity; or human transport in the ballast water of ships. The true mechanism cannot be elucidated from the work done thus far, but previous research on *A. tonsa* suggests the answer is complicated. It is know that *A. tonsa* do not thrive in the open ocean (Paffenhofer and Stearns 1988; Tester and Turner 1991), but how long they can survive has not been thoroughly investigated. Ballast water transport has been cited for the introduction of *A. tonsa* into Europe (Gaudy et al. 1995). However, ballast water transport of *A. tonsa* can be questioned because transport of ships between the naval shipyards in Narragansett Bay and Portsmouth Harbor (at the mouth of Great Bay) should have theoretically mixed these populations (unless the introduced individuals were unable to compete with the established population) but did not.

Whatever the mechanism of transport, is seems evident that the large population sizes and genetic diversity of estuarine copepods have left them poised to invade new territory when it becomes available. Lee (1999) has shown evidence of five separate and recent invasions of freshwater by the estuarine copepod *Eurytemora affinis*. In laboratory studies where samples of *E. affinis* are transitioned from salt to freshwater, most die, but some are able to survive and reproduce. On a less grand scale, most populations of estuarine copepods are probably able to rapidly adapt to environmental changes allowing them to not only invade new territories, but to quickly adapt to them.

Choice of genetic marker for phylogenetic inference:

The analyses of genetic and phylogenetic structure of *A. tonsa* have been made using sequence data from mtCOI, mt16s rDNA (Caudill and Bucklin 2004), and AFLP

markers. Phylogenetic analysis using mtCOI and mt16s rDNA generated similar results. mt16s rDNA is a good marker because it evolves quickly and can reveal large differences even among closely related populations. The disadvantage of mt16s rDNA is that it encodes a ribosomal RNA for which the evolutionary constraints are difficult to determine. Further, the mt16S rDNA gene is prone to insertion/deletion polymorphisms that can be highly variable but difficult to interpret. mtCOI is a protein coding gene making the analysis of the sequence data easier to understand and interpret. mtCOI was able to reveal lineage sorting (all NH individuals can be traced back to a single DNA sequence that is not shared by any of the other populations) between the NH population and the remaining samples. It was a good marker of population structure and phylogenetic reconstruction. Bucklin et al (2003) successfully used mtCOI in a study comparing the relationships among 34 species in ten genera of calanoid copepods.

Neither the mtCOI nor the mt16S rDNA sequence data could detect structure between Buzzards Bay and Narragansett Bay, but the AFLP data could. The AFLPs appeared to evolve faster than either gene and were polymorphic enough to elicit structure between the two very closely related estuaries. The level of structure they detected was small and indicates that there is very little restraint on the flow of genetic material between estuaries. The rapid evolution of the AFLP markers, resulting in possible saturation between the NC and MA/RI populations, is surprising. If the markers were evolving with little or no selective constraint, then their level of diversity would be similar or less than the level of diversity among the 4-fold degenerate mtCOI sites because the mitochondrial genome evolves faster than the nuclear genome (Brown et al, 1976). It is possible that the pre-selection of markers for ones that generate a large number of bands resulted in

markers that produce more variable bands that are not reflective of the genetic variation between populations (i.e. they could have amplified a repetitive portion of the genome that was variable within individuals).

***Acartia tonsa* as a bioindicator:**

Acartia tonsa has been much-used as a bioindicator species, especially for determining the toxicity of environmental pollutants (Sunda et al. 1987; Girling 1989; Bushong et al. 1990; Sunda et al. 1990; Table 2-8). However, if the goal is to standardize the results of these tests, then an effort needs to be made to ensure that the same species is used for each substance being tested. The high level of genetic variation seen between populations of *A. tonsa* in this study implies that the populations have had time to adapt to their local environment. Each environment has its own set of pollutants (i.e. there was a US Air Force base bordering Great Bay, NH that had known environmental contamination problems reported by the US Air Force (1990; 1999)) that the local population of *A. tonsa* may have evolved in response to.

Thus, there may be significant differences in the LD50 of two populations of *A. tonsa* tested for the same compound. Schizas and Chandler (Schizas et al. 2001) have demonstrated that different mitochondrial lineages of the harpacticoid copepod *Microarthridion littorale* have significantly different LD50 when exposed to a pesticide mixture. This caution holds for any comparison being made between populations of what is now considered the species *A. tonsa*. This warning, however, should not discourage the use of *A. tonsa* for such studies. Instead, it should add to and enhance understanding of the results of such studies.

Pollutant	Reference
Acid Waste	(Rose et al. 1977)
Ammonia	(Sullivan and Ritacco 1985)
Antibiotic metronidazole	(Lanzky and Halling-Soerensen 1997)
Bis(tributyltin) oxide	(U'Ren 1983)
Bromochloride, chlorine	(Roberts and Gleeson 1978)
Cadmium	(Toudal and Riisgaard 1987)
Cadmium, Copper, and Mercury	(Sosnowski and Gentile 1987)
Chlorine	(Roberts et al. 1975)
Chlorine	(Heinle and Beavan 1977)
Chlorine	(Abarnous 1982)
Chlorine and Bromine Chloride	(Bradley 1978)
Contaminated Sediment	(Pedersen et al. 1998)
Copper	(Sosnowski et al. 1979)
Estrogen	(Andersen et al. 1999)
Exploration and production chemicals	(Sverdrup et al. 2002)
Fuel Oil	(Berman and Heinle 1980)
Fuel Oil	(Hollister et al. 1980)
Fuel Oil	(Vargo 1980)
Fuel Oil	(Suderman and Marcus 2002)
Gamma-HCH	(Chen and Moehlenberg 1991)
Glyphosate-based herbicides	(Tsui and Chu 2003)
Heavy Metals	(McConaugha 1976b)
Insecticide	(Newell et al. 1981)
Insecticide	(Tester and Costlow 1981)
Insecticide	(Thompson and Tucker 1989)
Linear alkyl benzene sulfonate	(Christoffersen et al. 2003)
Metals	(Sunda et al. 1987)
Metals	(Hook and Fisher 2001)
Methodology	(Lee 1977)
Methodology	(Wen Yuh 1977)
Methodology	(Ward et al. 1979)
Methodology	(Weideborg et al. 1997)
Oil	(Girling 1989)
Pesticides	(Khattat and Farley 1976)
Power Plants	(Heinle 1976)
Suspended Soils	(Sherk et al. 1976)
Synthetic musk	(Wollenberger et al. 2003)
Tibutyltin oxide	(Johansen and Moehlenberg 1987)
Toxicants	(Roberts et al. 1982)
Tributyltin and linear alkylbenzene sulfonate	(Kusk and Petersen 1997)
Waste	(McConaugha 1976a)

Table 2-8: A partial list of pollutants that have been tested on *A. tonsa* with references.

CONCLUSIONS

This study shows evidence that populations *A. tonsa* in estuaries along the East Coast of the USA are highly diverse, have clear genetic structures, and in some instances are cryptic species. This implies that populations of *A. tonsa* are evolving independently of

each other and in response to their local environment. These results are part of a growing body of evidence suggesting that many estuarine organisms are part of isolated populations and isolated ecosystems. These systems could provide a unique model system for testing theories of speciation and evolutionary adaptation.

CHAPTER III

COMPARATIVE ANALYSIS OF VARIATION IN PHYSIOLOGICAL ACCLIMATION OF TWO POPULATIONS OF *ACARTIA TONSA* TO EXTREME TEMPERATURES

ABSTRACT

This study examines whether the genetic differences between the two putative species of the calanoid copepod *Acartia tonsa* have resulted in physiological differences in their response to temperature stress. Replicate cultures from the two locations were subjected to high and low temperature over multiple generations (respectively, 31°C for 10 generations, and 5° and 7°C for 3 generations). The response to the stress is measured in terms of number of adults and numbers of viable eggs produced. In the 31°C cultures, there were significantly more NC adults than NH adults each generation and there was no significant difference between numbers of viable eggs produced per culture. At 7°C, the number of adults in both the NH and NC cultures decreased over the first two generations; in the third generation, there were few to no adults and no dormant eggs. At 5°C, the NH cultures declined in number of adults over the first two generations; a few nauplii and many dormant eggs made up the third generation. At 5°C, the number of adults increased in NC cultures over the first two generations; replicates varied in the third generation, from a sharp increase in the number of adults, to a decrease, to near zero adults. These results suggest that *A. tonsa* populations from NH and NC are physiologically, and perhaps evolutionarily, different. However, other differences

between the two cultures (i.e. a detrimental or beneficial microorganism co-cultured with one population and not the other) could also explain the observed results.

INTRODUCTION

Populations of *Acartia tonsa* from Great Bay, NH and Beaufort Inlet, NC have been designated as putative cryptic species (Chapter 2). Whether these putative species' separate evolutionary paths have resulted in physiological differences is unknown. This study examines if cultures derived from these populations have different physiological responses to extreme temperature, an environmental factor that differs between the species' home estuaries. It also lays the groundwork to address the evolutionary forces behind these differences.

Temperature has a profound effect on organisms (Hochachka and Somero 2002) and plays a major role in determining the distribution and abundance of marine organisms, especially ectotherms (Dahlhoff and Somero 1993). Temperature was chosen as the environmental factor for this study because of its range variation among the estuaries from which *A. tonsa* was collected. Ice core samples indicate that mean global temperature has been changing at varying rates for at least the past 65 million years, and current climate data indicate that temperature is still changing (Clarke 1996), making it a factor to which populations must adapt. The N. Atlantic has experienced significant temperature variation over the past tens of thousands of years (Lambeck and Chappell 2001). Estuarine temperatures also show dramatic variation on interannual to seasonal time scales (Wares and Cunningham 2001). It has been shown that temperature affects

diffusion of micromolecules within the cytoplasm, enzyme activity, structural protein function, and lipid membranes (Guderley 1990). In order to adapt to temperature changes, organisms must conserve the structural integrity of macromolecules, provide adequate supplies of micromolecules, and maintain the appropriate rate and direction of metabolic flow (Hochachka and Somero 2002).

Organisms can adapt to changes in the temperature in a variety of ways and on both short and long-term time frames. Individuals can adapt to temperature by undergoing physiological changes (acclimation), such as changing the amount and type of proteins produced. For instance, the calanoid copepod *Calanus finmarchicus* shows a 4-fold increase in the expression of heat shock 70 (hsp70) in response to 30 minutes of thermal stress (Voznesensky et al. 2004). On longer time frames, populations can adapt to temperature through changes in the genetic composition of the population, a process known as evolutionary adaptation (see Hochachka and Somero 2002) . Since temperature changes seasonally in many environments, including estuaries, it is thought that evolutionary adaptation to temperature may be occurring through cyclical selection. Of interest is whether cyclical selection acting on a population whose life span is shorter than the temperature changes to which they must adapt, can affect the phenotypic plasticity of individuals. For example, the temperature of an environment varies from 0° to 25°C over the course of a year, while the life span of an organism is two months. Thus, a new generation will face temperatures never encountered by their parents. Several theoretical models have been proposed to address this question, and they generally predict that cyclical selections will favor the maintenance of genetic heterozygosity. These models have been difficult to test in real systems because their

predicted outcome is similar to the predicted outcome of populations evolving under neutral conditions (Hairston and Dillon 1990).

The goal of this study was to examine the variation in phenotypic plasticity between genetically distinct populations of *A. tonsa* from estuaries with significantly different temperature ranges. *Acartia tonsa* is an obligate estuarine calanoid copepod with a short life span, large population sizes, and wide distribution (see Chapter 1). These conditions make it an ideal organism for addressing questions about evolutionary adaptations to cyclical environments for several reasons. First, many of the environmental conditions in estuaries are variable, some factors vary sporadically over short time periods (i.e. salinity) while others vary more predictably over longer time periods (i.e. temperature). Second, populations with large effective population sizes (*A. tonsa* have been reported in excess 10^5 individuals per m^3 in Narragansett Bay (Durbin and Durbin 1981)) should theoretically be more responsive to selective forces than to random genetic drift (see Hartl and Clark 1989 chapter 4). Third, populations of *A. tonsa* are widely distributed with restricted migration between populations, thus, there could be many populations independently evolving to different environmental conditions.

MATERIALS AND METHODS

Sample collection:

Acartia tonsa adults were collected from Great Bay, NH (43°54.5"N; 70°51.8"W) on October 31, 2000 (NH collection) from the dock at the University of New Hampshire's

Jackson Laboratory in Durham, NH (water temperature = 9°C). Samples were collected with a 333μm mesh net using a one quart glass jar as a cod end. The net was tossed into the water, allowed to sink, and then was slowly pulled up and through the water column. The samples were filtered to remove large predators and debris and stored in a cooler with ice packs for transportation to UNH, where *A. tonsa* were identified and removed alive. Collections were also done from Gallants Channel, in Beaufort Inlet, in Beaufort NC (34°43'12"N; 76°40'23"W) on October 28, 2000 (NC collection) off the Pivers Island Road bridge (water temperature = 20°C). North Carolina samples were collected and sorted by Dr. Patricia Tester (National Oceanic and Atmospheric Administration, Southeast Fisheries Science Center). Copepods were fed, placed in a one-quart cooler, and shipped overnight to UNH. *Acartia tonsa* were identified by the morphology of the fifth thoracic leg of adult females, only adult females were identified from the collections.

Culturing and experimental setup:

In the laboratory, 20 – 30 fertilized females were placed in 2L glass bottles containing sea water collected from the same location and filtered through 20μm mesh (Figure 3-1). Three cultures were established for each collection, and grown at room temperature with a 14:10hr light:dark cycle. Every other day, 200ml of water was removed from each culture using a siphon fitted with a 20μm mesh and replaced with 200ml of 20ppt Instant Ocean containing a mixture of *Rhodomonas salina* (CCMP1319) and *Isochrysis galbana* (CCMP1323). Phytoplankton cultures were grown in Guillard's f/2-si media in 33ppt Instant Ocean at room temperature with a 14:10hr light:dark cycle.

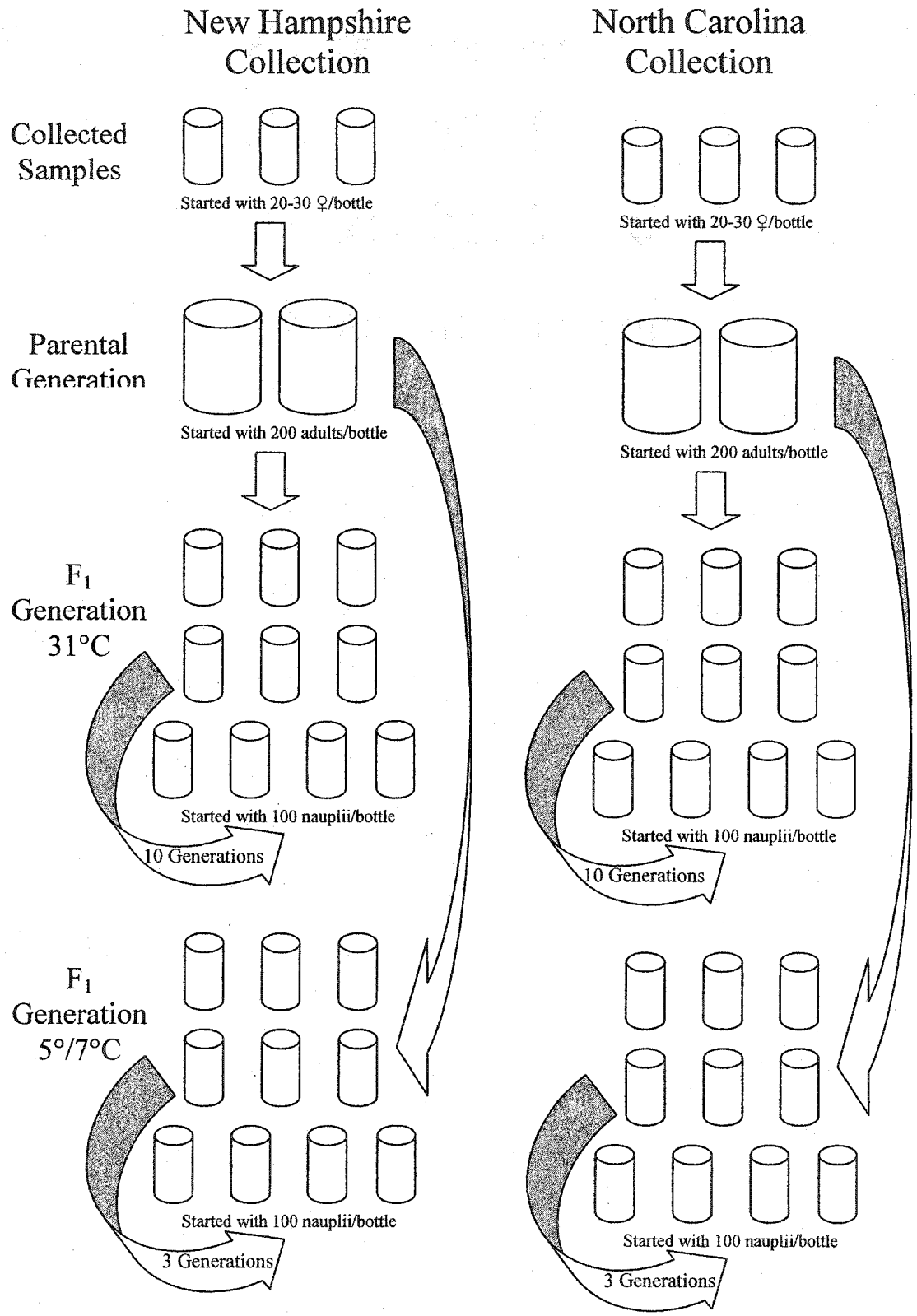

Figure 3-1: Experimental setup. Small cylinders = 2L bottles, large cylinders = 10L bottles, and arrows = transfer of *A. tonsa* between generations.

The parental generation for the experiment was started using 200 adult offspring of the collected samples to seed each of 2 NH and 2 NC cultures in 10L bottles. These cultures were grown at 20°C with a 14:10hr light:dark cycle. Every other day 1L of water was removed using a siphon fitted with a 20μm mesh and replaced with 1L of 20 ppt Instant Ocean containing mixed phytoplankton. Their offspring were used to set up four experimental treatments consisting of both NH and NC cultures at 31°and 5°C. Each treatment consisted of 10 replicate 2L experimental cultures, each seeded with 100 nauplii at 20°C. Once seeded, the temperature of the cultures was increased or decreased at a rate of 2°C/day until the treatment temperature was reached. The cultures were maintained at the treatment temperature (31°C or 5°C), with a 14:10hr light:dark cycle. Through an incubator problem, only 4 of the 5°C cultures from each collection site were at 5°C, the remaining 6 cultures were at 7°C. All experimental cultures were fed every other day by removing 200ml of water with a siphon fitted with a 20μl mesh and replacing it with 20ppt Instant Ocean containing mixed phytoplankton. During the experiment a new generation was defined as the time when nauplii were first observed in the bottle.

Observations and data collection:

During the experiment, the numbers of males and females were measured at the beginning of each generation by filtering each bottle through a 200μm mesh and into a fresh bottle. The adults were caught in the mesh and the males and females were separated and counted. Experiments to determine the number of viable eggs produced by females were conducted by placing six fertilized females from each culture into individual egg laying chambers with an adult male (for a total of 60 females per

treatment). Egg laying chambers consisted of a plastic vial with a 100μm mesh on the bottom and the other end open, placed in a 60ml jar. Adults placed in the plastic vial cannot get out, while the eggs fall through the mesh to the bottom of the jar. For the 31°C treatment, eggs were collected over a 3 day period in one chamber. Adults were moved to a new chamber and eggs were collected for another 3 day period. Eggs and nauplii were counted on days 3 and 4 (no eggs remained after day 4). For the 5° and 7°C treatments, eggs were collected for a 7 day period in one chamber. Adults were then moved to a new chamber and eggs were collected over another 7 day period. Eggs and nauplii were counted after 0, 3, 6, 9, and 12 weeks.

The 31°C cultures were maintained and monitored for 10 generations. The numbers of males and females were counted each generation. The number of nauplii produced per female was calculated at generations F_1, F_3, and F_{10}. The 5° and 7°C cultures were maintained and monitored for 3 generations. The numbers of males and females were counted each generation and the numbers of nauplii produced per female were calculated at generation F_1.

Statistical analysis:

Data for observations of both cultures over multiple generations were analyzed using multivariate profile analyses (Rencher 1995). Under this analysis, each generation was treated as a separate dependent variable and the culture was the independent variable. While this method has less power than the equivalent univariate method (split-plot ANOVA: von Ende 1993), it does not assume that the within subject factor (generation) is equally correlated at each level and is therefore less likely to give a false positive result

(Rencher 1995). When appropriate, the results were compared using a paired t-test or a 2-way ANOVA (Sokal and Rohlf 1995). For proportional data where the values fell below 0.3, the data were arcsine transformed, where $X = \sin^{-1}(((Y + 3/8)/(n + 3/4))^{1/2})$, where Y is the proportion, X is the transformed value in degrees, and n is the number of replicates (Sokal and Rohlf 1995).

Only cultures that survived the entire experiment were analyzed for comparisons between treatments. Neither the split-plot nor the profile analyses are able to handle missing data in the number of within-subject factors (von Ende 1993). Thus, including cultures that died before the conclusion of the experiment in this analysis would artificially increase the variance of the within-subject factors, without adding real meaning to the analysis, while potentially masking significant differences between the factors.

RESULTS

Population size at 31°C:

At 31°C *Acartia tonsa* populations in 6 of the 10 NH and 9 of the 10 NC bottles survived all 10 generations (Table 3-1). There was no significant difference in the number of bottles whose populations survived all 10 generations between the NH and NC cultures ($p = 0.1307$ by G-test).

The mean number of adults in the NH and NC cultures was similar in the F_1 generation (Table 3-1, Figure 3-2). Over the next 9 generations, the mean number of adults in the NH cultures remained relatively constant while the mean number of adults in the NC

31°C	Gen	F_1		F_2		F_3		F_4		F_5		F_6		F_7		F_8		F_9		F_{10}	
Culture	Bottle	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
NH	1	16	7	16	5	10	6	13	6	18	8	14	5	17	6	11	3	13	6	16	8
	2	14	6	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	10	7	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	15	9	18	5	19	10	12	2	16	6	17	8	8	2	12	6	17	5	14	7
	5	14	9	12	5	19	9	17	6	6	4	13	5	11	5	8	4	13	6	10	6
	6	7	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	15	7	19	8	21	10	25	14	18	7	16	7	18	9	21	11	16	5	21	13
	8	15	9	10	4	14	7	13	8	19	7	22	11	17	6	22	13	16	8	13	6
	9	22	8	26	12	21	7	23	10	16	9	24	7	13	7	18	12	22	12	21	8
	10	10	6	6	1	8	6	3	0	0	0	0	0	0	0	0	0	0	0	0	0
	Mean	*16.17*	*8.17*	*16.83*	*6.50*	*17.33*	*8.17*	*17.17*	*7.67*	*15.50*	*6.83*	*17.67*	*7.17*	*14.00*	*5.83*	*15.33*	*8.17*	*16.17*	*7.00*	*15.83*	*8.00*
	sd	*2.93*	*0.98*	*5.67*	*3.02*	*4.41*	*1.72*	*5.60*	*4.08*	*4.81*	*1.72*	*4.41*	*2.23*	*4.00*	*2.32*	*5.79*	*4.36*	*3.31*	*2.68*	*4.45*	*2.61*
	Mean	*24.33*		*23.33*		*25.50*		*24.83*		*22.33*		*24.83*		*19.83*		*23.50*		*23.17*		*23.83*	
	sd	*2.88*		*8.50*		*5.75*		*9.35*		*6.22*		*6.15*		*6.05*		*9.99*		*5.64*		*6.68*	
NC	1	11	7	20	7	40	9	52	8	25	6	55	9	37	9	29	7	47	10	44	10
	2	46	9	11	6	33	6	28	4	59	13	26	4	43	8	44	11	22	5	64	14
	3	11	7	42	10	48	13	44	6	48	8	44	11	49	12	36	9	30	7	98	9
	4	22	8	10	8	6	7	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	10	6	26	9	38	8	47	10	32	6	40	7	53	10	55	10	51	11	33	7
	6	16	8	36	7	42	9	52	5	42	7	48	8	34	8	38	7	37	10	44	9
	7	23	9	41	8	55	14	31	4	39	6	34	6	45	12	34	12	41	8	39	11
	8	17	9	38	5	41	10	45	9	49	9	41	8	65	15	49	9	36	9	43	8
	9	14	6	56	6	20	6	27	7	37	11	50	7	37	7	27	7	54	9	49	9
	10	38	10	50	5	38	11	42	14	31	7	24	6	42	12	45	6	43	12	52	8
	Mean	*20.67*	*7.89*	*35.56*	*7.00*	*39.44*	*9.56*	*40.89*	*7.44*	*40.22*	*8.11*	*40.22*	*7.33*	*45.00*	*10.33*	*39.67*	*8.67*	*40.11*	*9.00*	*51.78*	*9.44*
	sd	*12.88*	*1.45*	*14.32*	*1.73*	*9.67*	*2.79*	*9.80*	*3.24*	*10.54*	*2.47*	*10.57*	*2.00*	*9.63*	*2.60*	*9.30*	*2.06*	*10.15*	*2.12*	*19.39*	*2.07*
	Mean	*28.56*		*42.56*		*49.00*		*48.33*		*48.33*		*47.56*		*55.33*		*48.33*		*49.11*		*61.22*	
	sd	*14.01*		*14.21*		*12.20*		*11.35*		*12.44*		*12.05*		*11.81*		*10.05*		*11.79*		*19.97*	

Table 3-1: Number of females and males for each bottle at 31°C for each generation from the NH and NC cultures. Means and one standard deviation are given for females, males, and totals only for the bottles whose population survived all 10 generations. Data for bottles whose population died before the 10^{th} generation are highlighted.

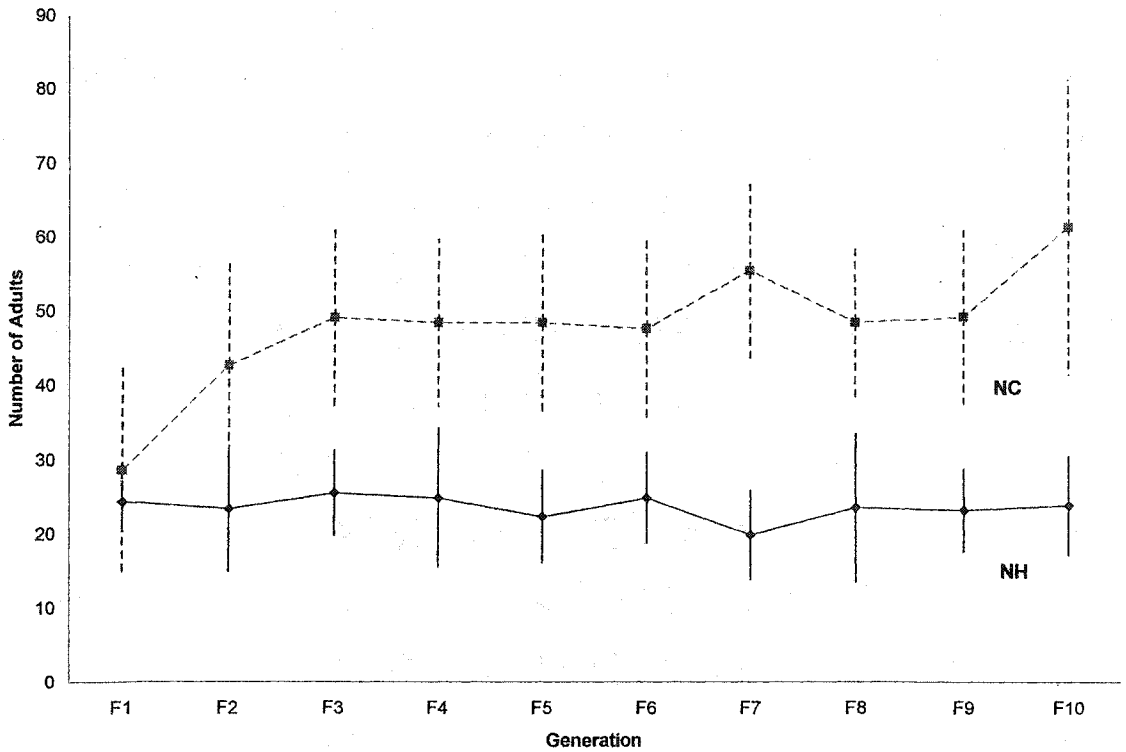

Figure 3-2: Mean of the total number of adults counted at 31°C in each bottle for each generation for the NH and NC cultures. Error bars are one standard deviation. Calculations only include bottles whose population survived all 10 generations.

cultures gradually increased (Figure 3-2). A multivariate profile analysis found a significant difference between the number of adults in the two cultures ($p < 0.0001$) and between generations ($p = 0.0397$) but no significant culture by generation interaction ($p = 0.0505$; Table 3-2).

Dividing the results by gender (Figure 3-3) showed that across all generations in both cultures the females significantly outnumbered the males ($p < 0.0025$ (0.05/20) by paired one tailed t-test for each culture at each generation; Table 3-3). Further, the females outnumbered the males within every bottle at every generation. It also showed that the observed increase in the NC culture was the result of an increase in the number of females which more than doubled over the 10 generations ($X = 20.67 \pm 12.88$ at F_1,

Population Size	Probability		
Comparison	Between Cultures	Between Generations	Culture by Generation
Total Number Adults			
NH 31°C vs NC 31°C	<0.0001**	0.0397*	.0505
NH 5°C vs NC 5°C	<0.0001**	<0.0001**	<0.0001**
NH 7°C vs NC 7°C	0.4018	0.1148	0.2489
Number of Males			
NH 31°C vs NC 31°C	0.0745	0.0561	0.0787
NH 5°C vs NC 5°C	<0.0001**	<0.0001**	<0.0001**
NH 7°C vs NC 7°C	0.7549	0.2015	0.7717
Number of Females			
NH 31°C vs NC 31°C	< 0.0001**	0.0923	0.1069
NH 5°C vs NC 5°C	<0.0001**	<0.0001**	<0.0001**
NH 7°C vs NC 7°C	0.3279	0.0954	0.1804

Table 3-2: Comparison of the NH and NC cultures across all 31°C generations and the 5°C and 7°C F_1 and F_2 generations for total number of adults per bottle, number of males per bottle, and number of females per bottle. Probabilities calculated using a multivariate profile analysis: Between Cultures is the probability that there was no difference between the NH and NC cultures; Between Generations is the probability that there was no difference between generations; and Culture by Generation is the probability that there is a culture by generation interaction. Data based on 6 bottles for the NH cultures and 9 bottles for the NC cultures. Only bottles whose population survived all 10 generations were included in the 31°C calculations. *significant, **highly significant.

31°C	Gen	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Culture	Bottle	*p*♀	*p*♀	*p*♀	*p*♀	*p*♀	*p*♀	*p*♀	*p*♀	*p*♀	*p*♀
NH31	1.00	0.70	0.76	0.63	0.68	0.69	0.74	0.74	0.79	0.68	0.67
	4.00	0.63	0.78	0.66	0.86	0.73	0.68	0.80	0.67	0.77	0.67
	5.00	0.61	0.71	0.68	0.74	0.60	0.72	0.69	0.67	0.68	0.63
	7.00	0.68	0.70	0.68	0.64	0.72	0.70	0.67	0.66	0.76	0.62
	8.00	0.63	0.71	0.67	0.62	0.73	0.67	0.74	0.63	0.67	0.68
	9.00	0.73	0.68	0.75	0.70	0.64	0.77	0.65	0.60	0.65	0.72
	Overall	*0.66*	*0.73*	*0.68*	*0.71*	*0.69*	*0.71*	*0.71*	*0.67*	*0.70*	*0.66*
	sd	*0.05*	*0.04*	*0.04*	*0.09*	*0.05*	*0.04*	*0.06*	*0.06*	*0.05*	*0.04*
NC31	1.00	0.61	0.74	0.82	0.87	0.81	0.86	0.80	0.81	0.82	0.81
	2.00	0.84	0.65	0.85	0.88	0.82	0.87	0.84	0.80	0.81	0.82
	3.00	0.61	0.81	0.79	0.88	0.86	0.80	0.80	0.80	0.81	0.92
	5.00	0.63	0.74	0.83	0.82	0.84	0.85	0.84	0.85	0.82	0.83
	6.00	0.67	0.84	0.82	0.91	0.86	0.86	0.81	0.84	0.79	0.83
	7.00	0.72	0.84	0.80	0.89	0.87	0.85	0.79	0.74	0.84	0.78
	8.00	0.65	0.88	0.80	0.83	0.84	0.84	0.81	0.84	0.80	0.84
	9.00	0.70	0.90	0.77	0.79	0.77	0.88	0.84	0.79	0.86	0.84
	10.00	0.79	0.91	0.78	0.75	0.82	0.80	0.78	0.88	0.78	0.87
	Overall	*0.69*	*0.81*	*0.80*	*0.85*	*0.83*	*0.84*	*0.81*	*0.82*	*0.82*	*0.84*
	sd	*0.08*	*0.09*	*0.03*	*0.05*	*0.03*	*0.03*	*0.02*	*0.04*	*0.02*	*0.04*

Table 3-3: Proportion of females in each bottle at 31°C from the NH and NC cultures. Overall is the portion of females from all bottles in the culture, sd is one standard deviation. Only bottles whose population survived all 10 generation are shown.

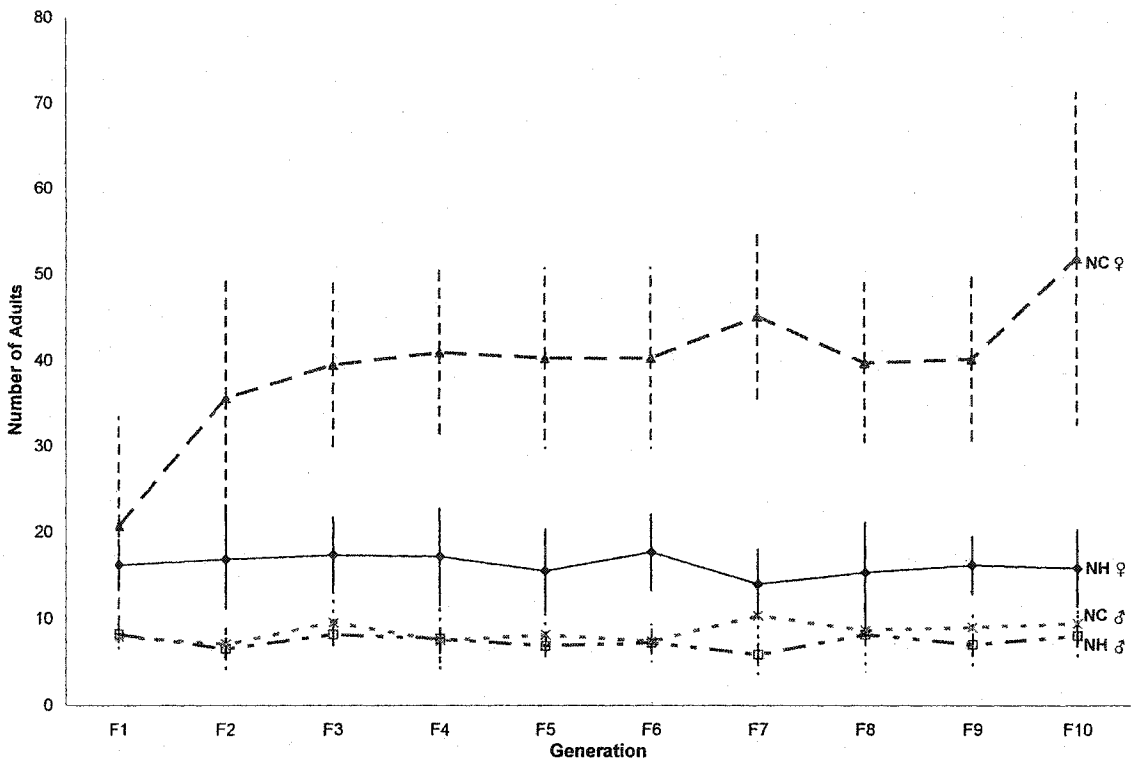

Figure 3-3: Mean number of adult females and males counted at 31°C in each bottle each generation from the NH and NC cultures. Error bars are one standard deviation. Calculations only include bottles whose population survived all 10 generations.

X=51.78±19.39 at F_{10}). The number of NC males and NH males and females remained relatively constant over 10 generations. There was a significant difference between the number of NH and NC females ($p < 0.0001$), but not between the number of NH and NC males ($p = 0.0745$). No significant difference was observed in the number of adults between generations for either the males or females (Table 3-2).

Nauplii production at 31°C:

The NH and NC cultures produced similar numbers of nauplii at all three generations tested (Figure 3-4, Table 3-4). The mean number of nauplii produced per female dropped between generations F_1 and F_3 and then rebounded by the F_{10} generation. The NH cultures produced more nauplii per female at all three generations, but the difference was

31°C	Gen	F_1			F_3			F_{10}		
Culture	Bottle	Mean	sd	*p*-Failed	Mean	sd	*p*-Failed	Mean	sd	*p*-Failed
NH31	1	31.17	13.29	0.00	14.50	8.89	0.00	27.00	23.39	0.00
	2	13.33	8.87	0.00	-	-	-	-	-	-
	3	17.17	9.58	0.00	-	-	-	-	-	-
	4	22.67	11.25	0.00	19.33	25.38	.017	18.67	21.99	0.00
	5	21.17	16.23	0.17	6.83	3.66	0.00	22.33	15.00	0.17
	6	11.50	16.16	0.50	-	-	-	-	-	-
	7	25.00	10.20	0.00	17.17	26.38	0.33	24.33	33.96	0.00
	8	5.00	6.03	0.50	16.17	18.71	0.00	25.50	16.22	0.17
	9	22.17	14.55	0.00	20.83	20.15	0.00	31.83	28.55	0.17
	10	24.67	13.65	0.00	6.00	8.37	0.33	-	-	-
	Mean	21.19	8.71	0.11	15.81	4.94	0.83	24.94	4.44	0.83
NC31	1	31.50	46.73	0.17	28.67	59.81	0.50	20.83	24.84	0.33
	2	14.83	16.13	.0.33	11.83	13.57	0.33	13.67	11.81	0.33
	3	18.17	15.56	0.33	10.17	13.23	0.33	18.17	22.33	0.33
	4	10.00	10.14	0.33	5.17	6.68	0.33	-	-	-
	5	16.17	16.86	0.33	11.17	11.39	0.33	17.33	20.33	0.33
	6	9.67	10.91	0.33	10.50	17.11	0.33	19.67	16.06	0.17
	7	15.50	15.91	.017	14.50	20.91	0.33	19.83	24.70	0.50
	8	33.00	17.11	.017	13.50	15.18	0.17	21.67	20.50	0.17
	9	20.67	20.31	.017	25.00	19.98	0.00	20.83	21.17	0.00
	10	22.67	10.07	0.00	15.67	19.79	.033	26.17	30.66	0.50
	Mean	20.24	7.75	0.22	15.67	6.65	0.30	19.80	3.40	0.30

Table 3-4: Results of 31°C nauplii production experiments at generations F_1, F_3, and F_{10} for the NH and NC cultures. The mean and standard deviation for each bottle are for 6 individuals per bottle. The p-Failed is the proportion of females per bottle that failed to produce nauplii. The per culture mean and standard error is the mean of means and standard deviation of means including only the bottles that survived all 10 generations. Dashes indicate no data available.

31°C Nauplii Production	Probability		
NH vs. NC Cultures	Between Cultures	Between Generations	Culture by Generation
Mean # Nauplii pre Bottle	0.3983	0.0079*	0.3229
Proportion ♀ Failed	0.0020*	0.8212	0.7017

Table 3-5: Comparison of the 31°C NH and NC cultures at the F_1, F_3, and F_{10} generations for mean number of nauplii produced per bottle and proportion of females who produced nauplii. The proportion data was arcsine transformed before calculations. Probabilities calculated using a multivariate profile analysis: Between Cultures is the probability that there was no difference between the NH and NC cultures; Between Generations is the probability that there was no difference between generations; and Culture by Generation is the probability that there is a culture by generation interaction. Data based on 6 females per bottle, 6 bottles for the NH cultures and 9 bottles for the NC cultures. Only bottles whose population survived all 10 generations were included in the calculations. *significant, **highly significant.

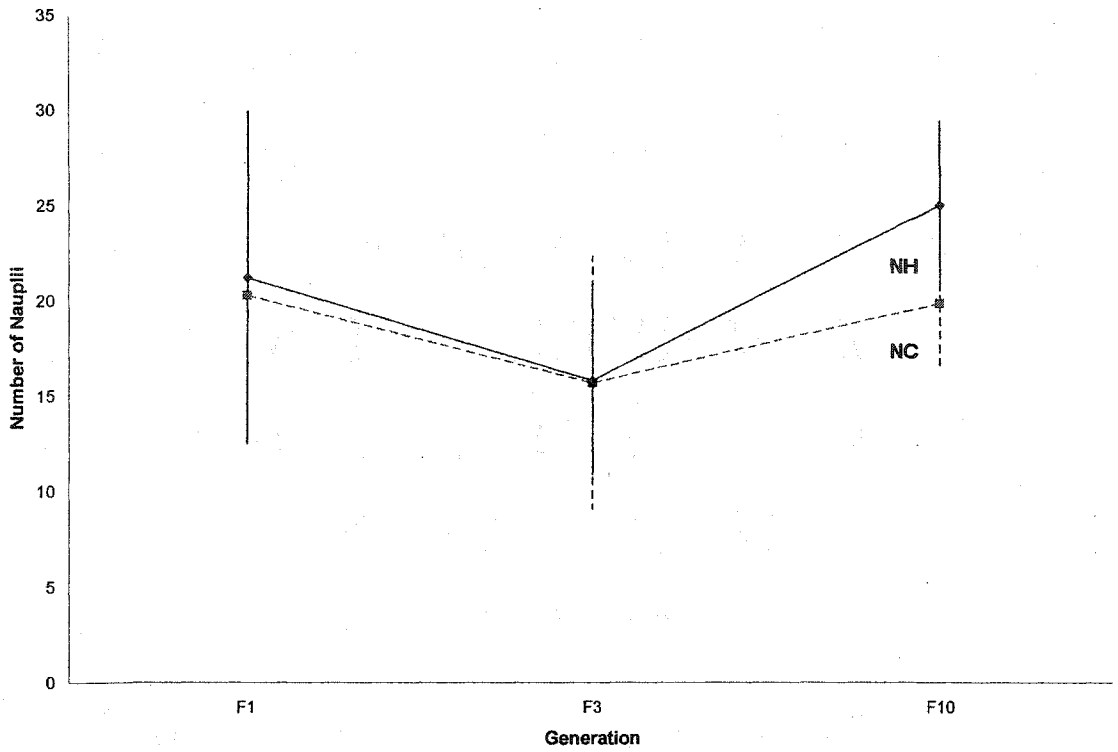

Figure 3-4: Mean of the mean number of nauplii produced per individual at 31°C for the NH and NC cultures at the F_1, F_3. and F_{10} generations. Error bars are one standard deviation of the means. Calculations only include bottles whose population survived all 10 generations.

insignificant ($p = 0.3983$). A significant difference in the number of nauplii produced per female between generations ($p = 0.0079$; Table 3-5) was observed.

There was a significant difference between the proportion of females that produced no nauplii between the two cultures ($p = 0.0020$) with a higher proportion of NC females failing to produce nauplii at all three generations (Table 3-4, Figure 3-5). Neither culture showed significant differences between generations ($p = 0.8212$; Table 3-5).

Population size at 5° and 7°C:

The cultures responded differently to the 5° and 7°C incubations, and all cultures showed variable responses at the F_3 generation (Table 3-6). The experiment was terminated after 8 weeks at the F_3 generations, because most of the cultures failed to generate any adults.

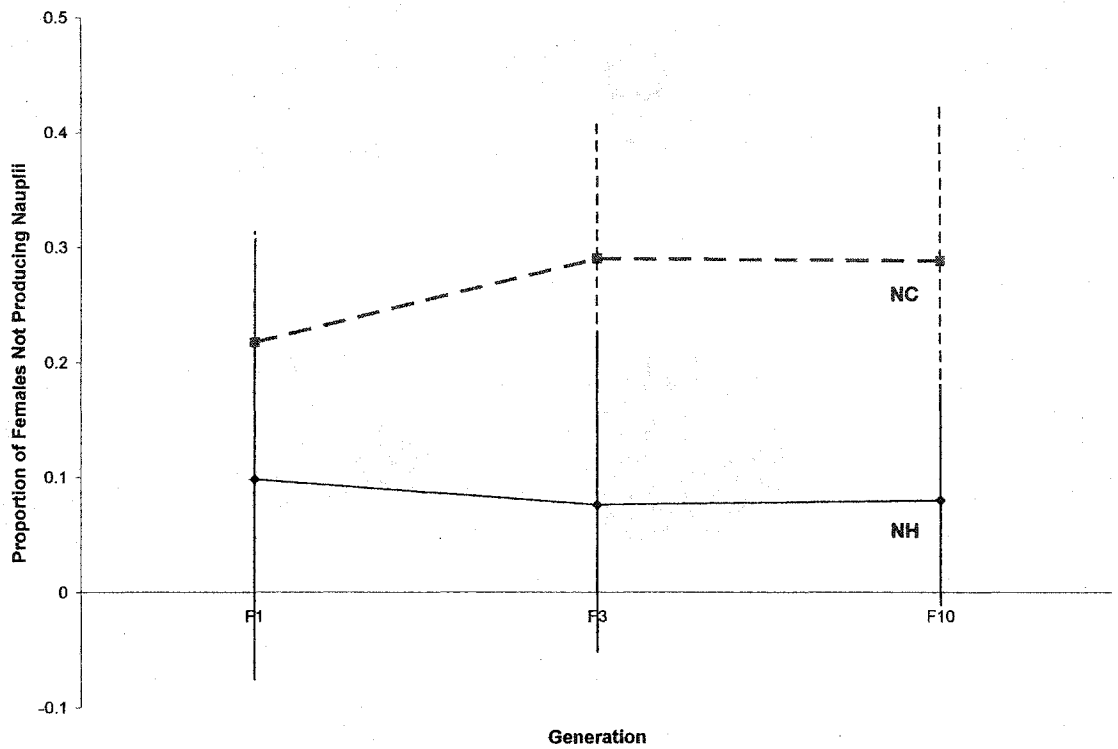

Figure 3-5: Mean proportion of females that produced no nauplii during the 31°C nauplii production experiment for the NH and NC cultures at generations F_1, F_3, and F_{10}. Error bars are 95% confidence limits. Means and confidence limits calculated after an arcsine transformation, data based on 6 females per bottle, 6 bottles for NH, and 9 bottles for NC. Only bottles whose populations survived all 10 generations were used in the calculations.

At 5°C, the NH cultures showed a slight decrease in the mean number of adults between the F_1 and F_2 generations (Figure 3-6); at the F_3 generation only eggs were produced, which hatched when brought to room temperature (Table 3-7). At 5°C, the NC cultures showed a sharp increase in the mean number of adults between the F_1 and F_2 generations (Figure 3-6); at the F_3 generation, 2 of the 4 bottles produced very few adults (14 total), while the other 2 bottles produced a large number of adults (a total of 839 for the 2 bottles; Table 3-7). At 7°C, the NH and NC cultures showed a decrease in the mean number of adults between the F_1 and F_2 generations (Figure 3-6); at the F_3 generation, nauplii were produced but no adults. None of the nauplii remaining in the bottles at the time of termination survived when brought to room temperature (Table 3-7).

5° and 7°C	Gen	F_1		F_2		F_3	
Culture	Bottle	♀	♂	♀	♂	♀	♂
	1	25	6	5	4	0	0
	2	37	5	1	1	0	0
	3	42	18	4	1	0	0
	4	34	22	32	22	0	0
NH	5	31	12	11	7	0	0
7°C	6	40	11	13	8	0	0
	Mean	*34.83*	*12.33*	*11.00*	*7.17*	*0.00*	*0.00*
	sd	*6.24*	*6.65*	*11.22*	*7.83*	*0.00*	*0.00*
	Mean	*47.17*		*18.17*		*0.00*	
	sd	*10.61*		*19.03*		*0.00*	
	1	36	15	27	9	0	0
	2	38	6	56	13	0	0
	3	34	21	35	13	0	0
NH	4	29	24	19	11	0	0
5°C	*Mean*	*34.25*	*16.50*	*34.25*	*11.50*	*0.00*	*0.00*
	sd	*3.86*	*7.94*	*15.90*	*1.91*	*0.00*	*0.00*
	Mean	*50.75*		*45.75*		*0.00*	
	sd	*4.79*		*17.21*		*0.00*	
	1	18	10	3	2	0	0
	2	17	12	5	3	2	2
	3	26	11	3	1	9	5
	4	15	7	62	25	0	2
NC	5	14	8	7	3	0	0
7°C	6	17	13	9	7	3	1
	Mean	*17.83*	*10.17*	*14.83*	*6.83*	*2.33*	*1.67*
	sd	*4.26*	*2.31*	*23.22*	*9.13*	*3.50*	*1.86*
	Mean	*28.00*		*21.67*		*4.00*	
	sd	*5.62*		*32.29*		*5.22*	
	1	35	27	149	92	1	0
	2	21	10	138	88	327	18
	3	29	18	79	70	12	1
NC	4	33	19	98	75	458	36
5°C	*Mean*	*29.50*	*18.50*	*116.00*	*81.25*	*199.50*	*13.75*
	sd	*6.19*	*6.95*	*32.99*	*10.44*	*229.23*	*16.98*
	Mean	*48.00*		*197.25*		*213.25*	
	sd	*12.94*		*43.42*		*245.85*	

Table 3-6: Number of females and males for each bottle at 5° and 7°C for each generation from the NH and NC cultures. Means and one standard deviation are given for females, males, and totals.

Culture	Bottle	Bottle Contents at time of termination				
		Adult ♀	Adult ♂	Nauplii	Eggs	Observations
NH 7°C	1	0	0	0	0	Nauplii had been observed in bottle, but none remained at termination
	2	0	0	~18	0	Moved nauplii to room temperature, all died as nauplii
	3	0	0	~32	0	Moved nauplii to room temperature, all died as nauplii
	4	0	0	~41	0	Moved nauplii to room temperature, all died as nauplii
	5	0	0	~37	0	Moved nauplii to room temperature, all died as nauplii
	6	0	0	~45	0	Moved nauplii to room temperature, 1 survived to adulthood
NH 5°C	1	0	0	0	Many (>300)	Moved eggs to room temperature, many hatched within 48hr, 20 separated and observed, 10 of the 20 survived to adulthood
	2	0	0	0	Many (>300)	Moved eggs to room temperature, many hatched within 48hr, 20 separated and observed, 12 of the 20 survived to adulthood
	3	0	0	0	Many (>300)	Moved eggs to room temperature, many hatched within 48hr, 20 separated and observed, 3 of the 20 survived to adulthood
	4	0	0	0	Many (>300)	Moved eggs to room temperature, many hatched within 48hr, 20 separated and observed, 3 of the 20 survived to adulthood
NC 7°C	1	0	0	0	0	Nauplii had been observed in bottle, but none remained at termination
	2	2	2	~9	Few (10-20)	Moved nauplii to room temperature, all died as nauplii
	3	9	5	0	Few (10-20)	Nauplii had been observed in bottle, but none remained at termination
	4	0	2	0	Few (10-20)	Nauplii had been observed in bottle, but none remained at termination
	5	0	0	0	Few (10-20)	Nauplii had been observed in bottle, but none remained at termination
	6	3	1	0	Few (10-20)	Nauplii had been observed in bottle, but none remained at termination
NC 5°C	1	1	0	>100	Few (10-20)	Separated 20 nauplii and moved to room temperature, 5 of the 20 survived to adulthood
	2	327	18	~3	Few (10-20)	Moved nauplii to room temperature, all died as nauplii
	3	12	1	~12	Few (10-20)	Moved nauplii to room temperature, all died as nauplii
	4	458	36	0	Few (10-20)	

Table 3-7: Contents of the 5° and 7°C NH and NC cultures after 8 weeks at the F_3 generation.

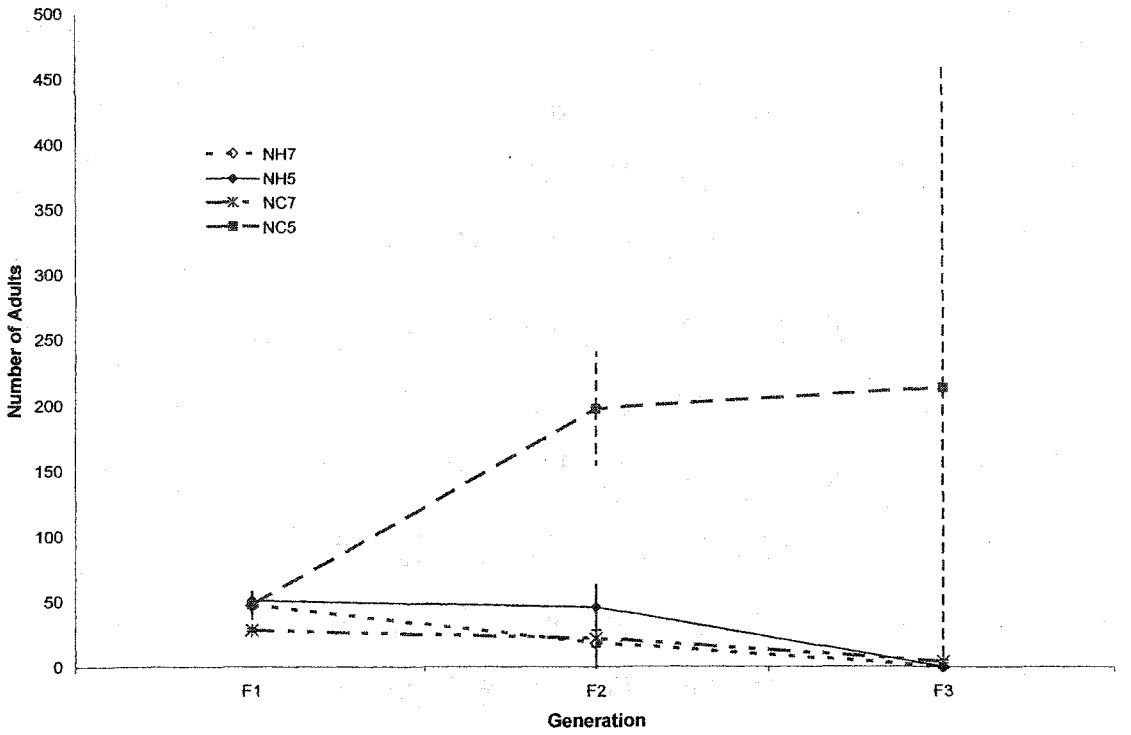

Figure 3-6: Mean of the total number of adults counted at 5° and 7°C in each bottle for each generation for the NH and NC cultures. Error bars are one standard deviation.

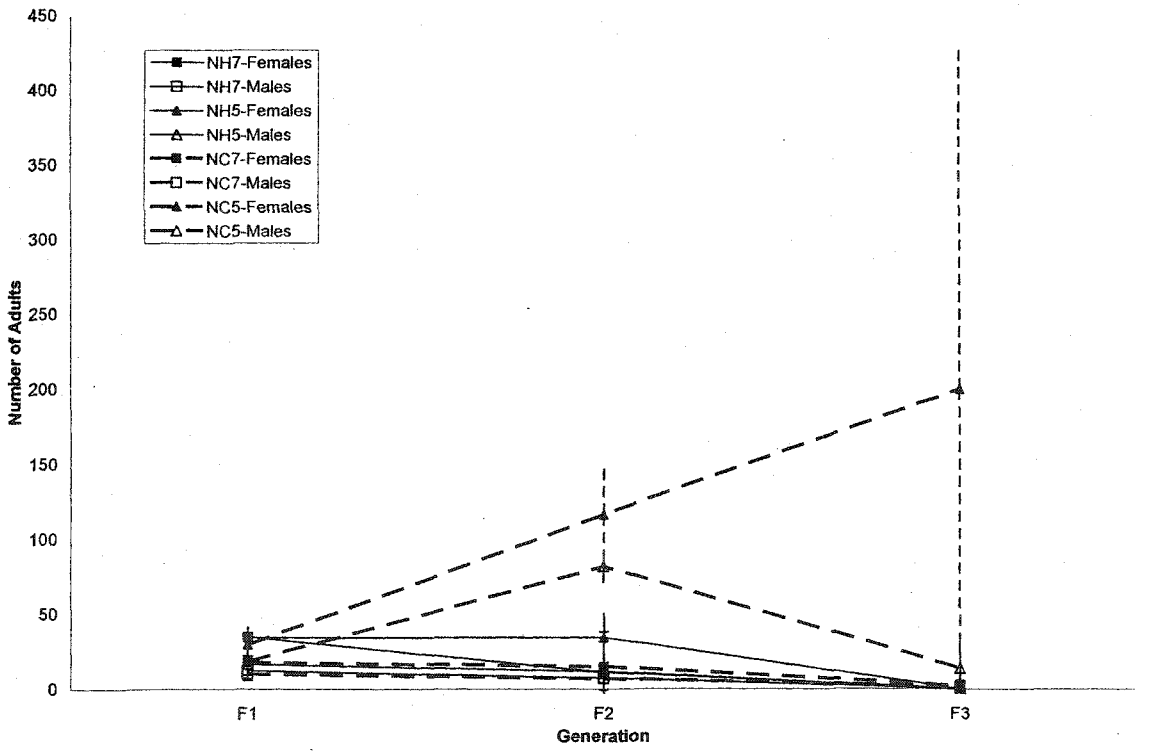

Figure 3-7: Mean number of males and females counted at 5° and 7°C for the NH and NC cultures. Error bars are one standard deviation.

Females outnumbered males in every bottle at all three generations for both temperatures and both cultures, with one exception (bottle 4 of the F_3 generation for the 7°C NC culture where there were no females and 2 males; Figure 3-7). This difference was significant for NH and NC at 7°C and NC at 5°C for the F_1 generation (using a paired t-test on each culture for each generation and temperature with an adjusted critical p value of 0.005 to compensate for multiple comparisons). The difference in the ratio of females to males was the largest in the two NC 5°C F_3 bottles that produced large numbers of adults, bottle 2 had a ratio of 18.17:1 and bottle 4 had a ratio of 12.72:1 (Table 3-7).

Nauplii production at 5 and 7°C:

The NH 7°C cultures produced significantly more nauplii per female than the NC 7°C cultures ($p < 0.0001$ by ANOVA); and significantly fewer NH females failed to produce nauplii ($p < 0.0001$ by ANOVA after arcsine transformation; Table 3-8). The NH and NC 5°C cultures produced similar mean numbers of nauplii per female and had a similar proportion of females fail to produce nauplii (Table 3-8). The higher variance in the NC 5°C cultures resulted in a significant culture by bottle interaction on a two way ANOVA ($p = 0.0259$) preventing a direct comparison of the means.

DISCUSSION

Responses of *Acartia tonsa* cultures to high and low temperatures:

The significant differences in the responses of the NH and NC cultures to high and low temperatures suggest that the populations have differentially evolved, however, other

5° and 7°C	Gen	F_1		
Culture	Bottle	Mean	sd	p-Failed
NH 7°C	1	16.17	9.20	0.00
	2	9.33	11.52	0.17
	3	10.83	10.23	0.00
	4	20.17	13.38	0.00
	5	11.33	6.25	0.00
	6	20.00	5.62	0.00
	Mean	*14.64*	*3.01*	*0.07*
NH 5°C	1	17.00	3.95	0.00
	2	10.67	12.39	0.17
	3	17.50	6.16	0.00
	4	24.33	10.21	0.00
	Mean	*17.38*	*3.82*	*0.08*
NC 7°C	1	2.67	2.94	0.33
	2	2.00	4.43	0.67
	3	0.00	0.00	1.00
	4	1.00	2.00	0.67
	5	1.17	1.94	0.50
	6	0.00	0.00	1.00
	Mean	*1.14*	*1.72*	*0.27*
NC 5°C	1	9.00	12.65	0.33
	2	32.00	23.78	0.00
	3	10.33	9.44	0.00
	4	18.67	16.72	0.17
	Mean	*17.50*	*6.19*	*0.16*

Table 3-8: Results of 5° and 7°C nauplii production experiment at generation F_1 for the NH and NC cultures. The mean and standard deviation for each bottle are for 6 individuals per bottle. The p-Failed is the proportion of females per bottle that failed to produce nauplii. The per culture mean and standard error are the mean of means and standard deviation of means.

factors may have caused the observed differential response. The small number of adults observed in the cultures at high temperatures were consistent with the reduction in population size of natural populations at high temperature (Heinle 1969 and Pat Tester, National Oceanic and Atmospheric Administration, Southeast Fisheries Science Center, personal communication). The die-off of the cultures at 7°C is not consistent with what has been observed in natural populations of *A. tonsa* species at low temperatures, which is to reduce population size and lay dormant eggs (Durbin and Durbin 1981; Sullivan and McManus 1986). The response could be the result of the unnatural conditions

encountered in the cultures (i.e. low temperature with long day length and plentiful food supply). It is possible that some of the organisms systems were responding to one environmental cue, while others were responding to a different cue. The NH cultures at 5°C may argue against this theory since the cultures' behavior mirrored that of natural populations, which is to lay dormant eggs. This could be a threshold response where at 5°C, the organism will respond to temperature, but at 7°C the organism still response to other environmental cues. The NC cultures' response to 5°C may reflect a response to unnatural conditions, since the source population from which these cultures were derived rarely falls below 10°C. It is clear, however, that the NH and NC cultures did respond differently at 5°C, and that these differences were maintained over multiple generations. Perhaps the most striking difference is that the NH cultures laid dormant eggs while the NC cultures did not.

In addition to the significant differences in the number of adults between the NH and NC cultures at 31°C, there were also significant differences in the numbers of adults between generations. This suggests that population sizes of the two cultures may not be changing at the same rate over the ten generations. Indeed, the NC cultures showed a trend of increasing number of adults each generation, which was not seen in the NH cultures. These results do not have the power to significantly confirm this trend, but they suggest that selection may have acted on the NC cultures over the 10 generations of the experiment.

These findings suggest that the forces that differentiate the NH and NC cultures are acting on the developmental (NI to CV) stages of the copepods life history. The NH cultures produced an equal number of nauplii per females and had a higher proportion of

females producing nauplii that did the NC cultures. In order for the NC cultures to generate a greater number of adults, more of the NC nauplii must survive to adulthood. Also of interest is that most of the increase in the number of adults for the NC cultures resulted from an increase in the number of females. This suggests that selection has favored NC females and acted during the developmental stages, although more experiments are needed to confirm these observations.

The cultures that produced the largest increase in population size over multiple generations to high (NC at 31°C) and low (NC at 5°C) temperatures both did so by increasing the proportion of females. These results are consistent with those of Heinle who found that the sex ratio of *A. tonsa* from Chesapeake Bay was predominantly female under density stress (Heinle 1972) and heavy predation stress (Heinle 1970). Durbin and Durbin (Durbin and Durbin 1981) found that females made up about 60% of wild populations in Narragansett Bay under normal conditions. An increase in the proportion of females could be an adaptation to increase population size, as one male could fertilize many females.

Additional sources of differences between cultures:

It is not possible from this study to conclude that the differences observed between the cultures were due to a genetic effect derived from differential evolution of the source populations. Other factors, such as microorganisms co-cultured with the *A. tonsa*, could have conferred a selective advantage or disadvantage onto the individuals in one culture, generating the differences observed. This scenario could also explain the large amount of variance observed within some of the treatments (i.e. NC cultures at 5°C). It is also

important to note that even if the observed differences were the result of differential evolution between the two cultures, the evolutionary response may not have been a direct response to temperature. The cultures could have had differential responses to food, salinity, photoperiod, dissolved oxygen level, microbial makeup of the culture (even if it was the same for both cultures), or a number of other factors or combination of these factors. Indeed, the extreme temperature may have served to compound the response to one or more of these factors. Many aspects of *A. tonsa's* response to temperature warrant further study. A detailed examination of the acclimation of individuals from different populations to a variety of temperatures would provide insight into how temperature affects each stage of development.

Possible genetic mechanisms:

A major difficulty in detecting evidence of cyclical selection has been that the predicted outcome of cyclical selection (maintenance of heterozygosity) is similar to the predicted outcome of genetic drift (Lynch 1987). Several models of the predicted heterozygosity have been proposed under various assumptions (Wright 1948; Hedrick et al. 1976; Kirzhner et al. 1996; Korol et al. 1996; Kirzhner et al. 1998; Burger 1999). Some of these studies have suggested evidence of cyclical selection acting on particular populations (Hairston and Dillon 1990 and references therein). If the variation in response to extreme temperature of the cultures in the present study was due to differential evolution, then this suggest a method for detecting cyclical selection: examination of the physiological response of several closely related populations that have been isolated to environments with different ranges of a cyclical environmental factor. In the current study, temperature was examined, which is cyclical in both the NH and NC

estuaries with different ranges. Indeed, the cultures derived from these two estuaries followed the *a priori* prediction that at high temperatures, the NC cultures would have greater population sizes than the NH cultures. There are many isolated populations of *A. tonsa*, covering a large variation in temperature ranges, which would provide opportunity to test this hypothesis. Similar methods have been used to find evidence of directional selection, the difference here is to distinguish cyclical selection from directional selection by examining molecular patterns of heterozygosity. A correlation between the maximum temperature range of an estuary and the fitness of a population at high temperatures, combined with evidence of high molecular heterozygosity, would offer strong evidence of cyclical selection. The study could be further strengthened by examining physiological responses of cultured populations to an environmental factor that does not vary between populations, or varies sporadically for which no trend is expected. Such a study would require using closely related populations or species, which have large isolated populations, are easy to culture, and are exposed to a cyclical environment.

The current study cannot resolve the underlying molecular evolutionary mechanisms (if there are any) driving the observed changes. It has been theorized that a selective evolutionary response over a short number of generations may be caused by genetic rearrangements. Thus, selection under fluctuating environments may influence the evolution of recombination rates (Zhuchenko et al. 1985; Korol et al. 1998). However, studies of *Drosophila melanogaster* found no reduction in the rate of adaptation to artificial selection on a polygenic trait when chromosomal recombination was inhibited (Bourguet et al. 2003). We cannot know whether the different responses of the NH and NC cultures to high temperature resulted from the accumulation of adaptive alleles, or

other genetic mechanism. Additional experiments with more replicates and larger culture sizes carried out for longer periods are needed to confirm the trends seen here.

CONCLUSIONS

It is suggested that *Acartia tonsa* collected from coastal estuaries in NH and NC have significant physiological differences, despite their highly conserved morphologies. Previous population genetic analysis and breeding experiments suggest that these populations are genetically divergent but morphologically cryptic species. This study implies that both species have maintained enough phenotypic plasticity to acclimate to the cyclical and sporadic changes in temperature that are typical of coastal estuaries. *Acartia tonsa* provides a unique model system for studying the processes responsible for the evolution of phenotypic plasticity in response to cyclical selection, an important aspect of the mechanisms of evolution.

CHAPTER IV

COMPARATIVE ANALYSIS OF DORMANT EGGS PRODUCED BY TWO GENETICALLY DISTINCT POPULATIONS OF *ACARTIA TONSA*

ABSTRACT

The calanoid copepod *Acartia tonsa* over-winters as a dormant egg in estuaries of the Northeastern USA. This study examines whether these dormant eggs are in diapause (i.e., predisposed to become dormant in response to a non-limiting environmental cue) or are quiescent (i.e., dormant in response to a limiting environmental condition). An important distinction between diapause and quiescent eggs is that diapause eggs can remain dormant for multiple seasons, resulting in a reservoir of unhatched eggs in the sediments, or egg bank, which may have the effect of slowing the rate of evolution of a population. Diapause eggs usually require incubation in the dormant state before becoming competent to hatch. Eggs laid by *A. tonsa* cultures, derived from Great Bay, NH and Beaufort Inlet, NC, under laboratory conditions at 3° and 0°C with a short photoperiod were capable of hatching if brought to 20°C immediately after being laid. In addition, 60.8% of NH eggs laid at 3°C hatched during a 16 week incubation at 3°C (89.5% within the first week), and 67.5% of NC eggs laid at 3°C hatched during a 16 week incubation at 3°C (88.9% within the first week). These results demonstrate that *A. tonsa* from NH and NC produced quiescent rather than diapause eggs in laboratory cultures at low temperatures with a short photoperiod, and further suggests that these

populations may not produce an egg bank in the wild. These findings are consistent with studies showing high levels of genetic diversity and significant population structuring, consistent with rapid evolution.

INTRODUCTION

Danks (1987) defined dormancy as a "state of suppressed development," the extremes being diapause and quiescence. He characterized diapause as a programmed delay in development in response to a non-limiting environmental cue. Thus, an organism might not be adversely affected by short day lengths, but enters a dormant state when the days become shorter than 8 hours. Once an organism has entered diapause, there is usually a minimum time that it must remain in diapause before resuming normal development. In contrast, quiescence is characterized as a delay in development resulting from an immediate response to adverse environmental conditions (Danks, 1987). The quiescent state lasts only as long as the adverse conditions persist. Diapause eggs differ from quiescent eggs, in that the latter will immediately continue normal development when conditions become favorable, diapause eggs must first receive an environmental cue to break the diapause state, and require an incubation period prior to being susceptible to such a cue (Marcus 1996).

Many animals enter a dormant stage to survive conditions that are unfavorable to them. Dormancy allows the animal to focus its adaptive abilities to a limited range of environmental conditions. Temperature is a common factor forcing animals into

dormancy. In marine estuaries, annual temperatures can fluctuate significantly season to season. Planktonic invertebrates that can neither escape the estuary nor control their own temperature, they must adapt to or tolerate the water temperature variation – or enter a dormant state when the temperature is too hot or to cold.

Of the 1800 plus species making up the 41 families of the copepod order Calanoida, 49 species of five families have the ability to lay dormant eggs (Engel and Hirche 2004). The primary environmental cues that induce copepods to lay dormant eggs are population density, photoperiod, and temperature (Mauchline 1998). In-depth studies of the factors that induce diapause have only been conducted on a few species of marine copepods. Culturing experiments on *Labidocera aestiva* (which over-winter as diapause eggs) have shown that the most important environmental cue for diapause induction was photoperiod modified by temperature (Marcus 1980; Marcus 1982a; Marcus 1982b; Marcus 1984). Culture experiments have shown that *A. hudsonica* produces diapause eggs in response to high temperatures without regard to photoperiod (Sullivan and McManus 1986; Avery 1999) and *A. bifiosa* produced diapause eggs in response to photoperiod modified by temperature (Chinnery and Williams 2003).

In research cultures, it is important to distinguish the difference between quiescent and diapause eggs, because it can have a major effect on development times and responses to environmental factors. In the wild, diapause eggs can influence the rate and direction of evolution. Hairston and De Stasio (1988) showed that diapause eggs laid by the fresh water copepod *Diaptomus sanguineus* continue to hatch at least three years after being laid and can make up a significant portion of the gene pool over multiple years. This

temporal migration of alleles can slow the rate of evolution. The magnitude of this effect depends on how long the eggs can survive in the sediment and the rate at which they hatch out (Marcus 1996). These variables are not known for most species, but Marcus et al. (1994) found viable eggs buried in sediment that were at least 40 years old.

Within the family Acartiidae, at least 15 species are known to lay dormant eggs (Mauchline 1998). Two of these dominate the water column in estuaries of the Northeastern USA: *Acartia tonsa* in the spring and summer; and *A. hudsonica* in the fall and winter (Lee and McAlice 1979; Durbin and Durbin 1981; Sullivan and McManus 1986). In estuaries south of New Jersey, *A. tonsa* remains in the water year-round (Zillioux and Gonzalez 1972; McAlice 1981, and references there in). *Acartia tonsa* was the first marine copepod shown to be capable of laying dormant (Zillioux and Gonzalez 1972), this study, conducted on *A. tonsa* cultures from Narragansett Bay RI and Biscayne Bay FL, did not directly test whether the eggs were quiescent or diapause. Several other studies have suggested that *A. tonsa* lay quiescent eggs, but none have directly tested it: Uye and Fleminger (1976) examined the effect of temperature on egg hatching in *A. tonsa* from Northern California - though these samples may have been what is now referred to as *A. californiensis* (Trinast 1976; Trujillo-Ortiz 1990; Caudill and Bucklin 2004) and concluded that their cultures failed to lay diapause eggs; Chen and Marcus (1997) examined field caught eggs and eggs laid by field caught *A. tonsa* females from the northern Gulf of Mexico, and concluded that these populations lay quiescent eggs.

This study examines whether laboratory cultures of *A. tonsa* derived from populations from Great Bay NH and Beaufort Inlet NC laid diapause or quiescent eggs at low

temperatures with a short photoperiod. These eggs were identified as quiescent or diapause by examining whether an incubation period was needed to break dormancy.

MATERIALS AND METHODS

Sample collection:

Acartia tonsa adults were collected from Great Bay, NH (43°54.5"N; 70°51.8"W) on October 31, 2000 (NH collection) from the dock at the University of New Hampshire's Jackson Laboratory in Durham, NH. Samples were collected with a 333μm mesh net using a one-quart glass jar as a cod end. The net was tossed into the water, allowed to sink, and then was slowly pulled up and through the water column. The samples were filtered to remove large predators and debris and stored in a cooler with ice packs for transportation to UNH, where *A. tonsa* were identified and removed alive. Collections were also done from Gallants Channel, in Beaufort Inlet, near Beaufort, NC (34°43'12"N; 76°40'23"W) on October 28, 2000 off the Pivers Island Road bridge (NC collection). NC samples were collected and sorted by Dr. Patricia Tester (National Oceanic and Atmospheric Administration, Southeast Fisheries Science Center). Copepods were fed, placed in a one-quart cooler, and shipped overnight to UNH.

Culturing and experimental setup:

In the laboratory, 20 – 30 fertilized females were placed in 2L glass bottles containing sea water collected from the same location and filtered through 20μm mesh (Figure 4-1).

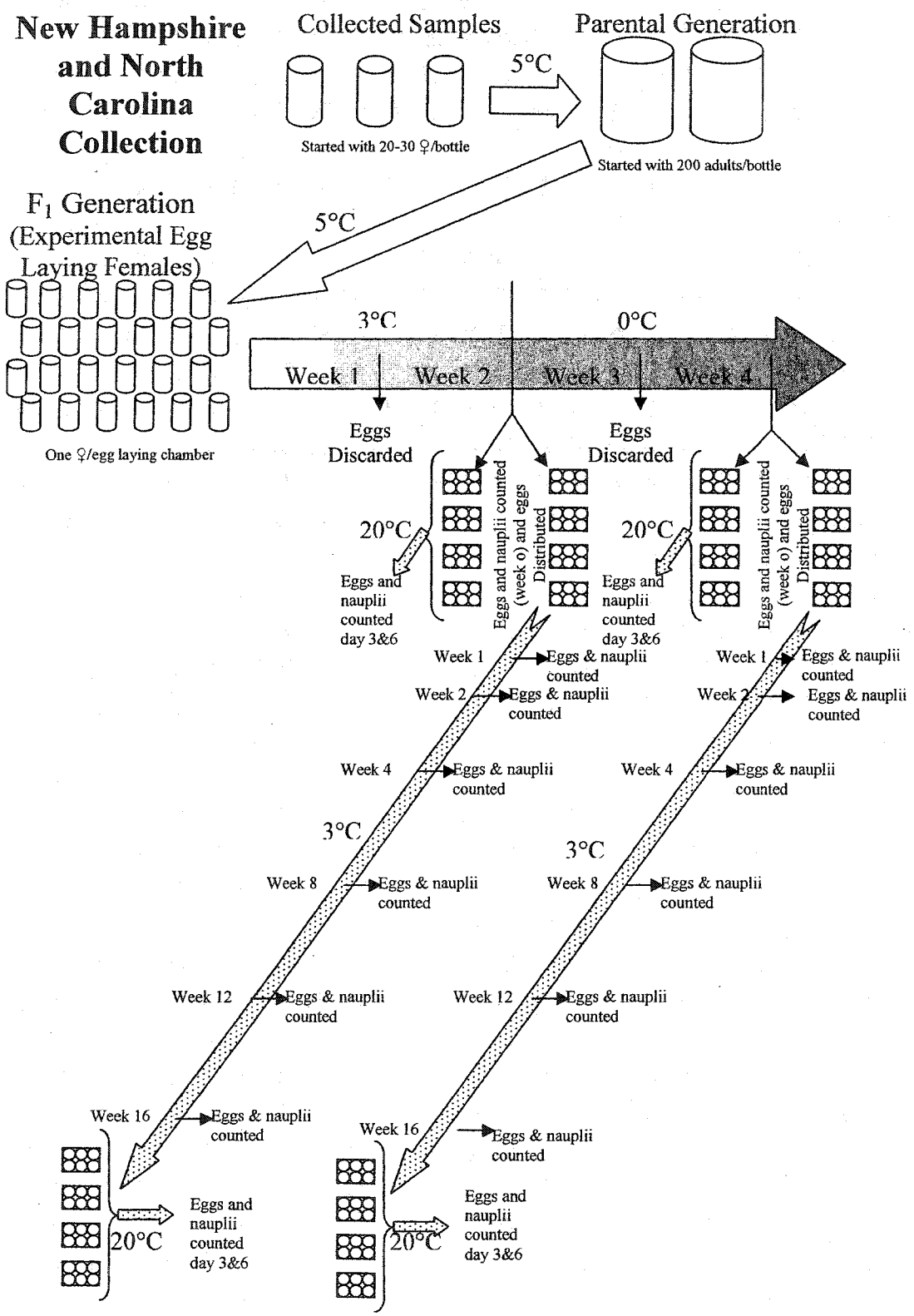

Figure 4-1: Experimental design. Large bottles are 10L, medium bottles are 2L, small bottles are 60ml, and boxes with circles are 6-well plates. Open arrows represent transfer between generations, patterned arrows represents time, wide patterned arrows are egg laying incubations, thin patterned arrows are egg hatching incubations, solid line arrows are egg and nauplii count points. Temperature of each incubation indicated.

Three cultures were established for each collection and grown at room temperature with a 14:10hr light:dark cycle. Every other day, 200ml of water was removed from each culture using a siphon fitted with a 20μm mesh and replaced with 200ml of 20ppt Instant Ocean containing a mixture of *Rhodomonas salina* (CCMP1319) and *Isochrysis galbana* (CCMP1323). Phytoplankton cultures were grown in Guillard's f/2-si media in 33ppt Instant Ocean at room temperature with a 14:10hr light:dark cycle.

Twenty fertilized female offspring of the field caught individuals were used to establish the parental generations in two 2L glass bottle for each of the NH and NC cultures. The bottles contained 20ppt Instant Ocean and were fed as described above. The temperature of the bottles was lowered from 20°C to 5°C at a rate of 2°C/day and the photoperiod was changed to a 6:18hr light:dark cycle.

The F_1 generation was used to seed the egg laying chambers, consisting of plastic vials with a 100μm mesh on the bottom and an open top, placed in a 60ml jar with 20ppt Instant Ocean. The adults were retained within the plastic vial but the eggs could fall through the mesh to the bottom of the jar. Twenty-four egg laying chambers each were established for the NH and NC cultures, each with one fertilized female and one male per chamber. Cultures were fed every other day by replacing 3ml of water with 3ml of 20ppt Instant Ocean and mixed phytoplankton, and were incubated with a 6:18hr light:dark photoperiod. The chambers were incubated at 3°C for two weeks. Eggs laid during the first week were discarded. After the second week, all of the eggs laid were collected and placed into the wells of clear plastic 6-well plates. The eggs collected from each chamber were split between two wells of separate plates to form 2 sets of plates, each set

containing eggs from every egg laying chamber in individual wells. The incubation temperature of the egg laying chambers was then lowered to 0°C and the experiment was continued for two more weeks. After the first week at 0°C all eggs laid were discarded. After the second week at 0°C, all of the eggs laid were collected and placed into the wells of clear plastic 6-well plates as before.

One set of plates from the 3°C incubation and from the 0°C incubation were immediately brought to 20°C immediately after being collected. The numbers of eggs and nauplii in each well of each plate were counted after 3 and 6 days (all eggs had hatched or dissolved by day 6) at 20°C. The other sets of plates were incubated at 3°C and the number of eggs and nauplii in each well of each plate were counted after 1, 2, 4, 8, 12, and 16 weeks. After 16 weeks at 3°C the temperature of the plates was raised to 20°C and the number of eggs and nauplii in each well of each plate were counted after 3 and 6 days (all eggs had hatched or dissolved by day 6) at 20°C.

Statistical analysis:

Percentage data were analyzed using nonparametric rank tests: Mann-Whitney test for non-paired two sample comparisons (Zar 1996, Chapter 8), Wilcoxon test for paired samples (Zar 1996, Chapter 9), and Friedman's test for analysis of variance with nonparametric Tukey-type test for multiple comparisons (Zar 1996, Chapter 12). Counts were square-root transformed prior to calculations of means and confidence limits (Zar 1996, Chapter 13).

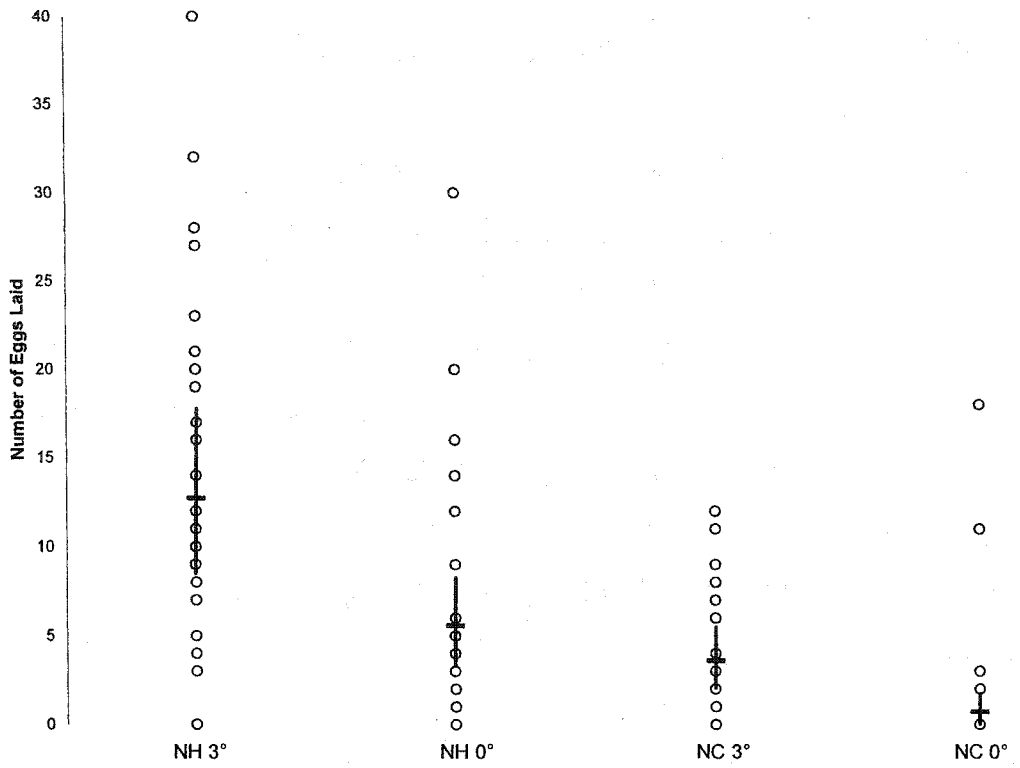

Figure 4-2: Number of eggs laid by each female over the 1-week incubation at 3° or 0°C from NH and NC. Open circles are values for each female (24/group), cross bar is at mean, and error bar represents 95% confidence limits. Means and confidence limits were calculated using square root transformed data.

RESULTS

The NH and NC females produced viable eggs during the 16-week incubation at 3° and 0°C (Figure 4-2, Tables 4-1 and 4-2). More eggs per female were laid at 3°C than 0°C within each culture, and the NH females laid more eggs per female than NC females at a given temperature (Table 4-3). At both temperatures, more NH females than NC females laid viable eggs with only one of the 24 NC females producing viable eggs at 0°C (Table 4-3). For the NH eggs laid at 3° and 0°C, there was no significant difference between the percentage of eggs hatched during the 20°C incubations at week 0 and week 16 ($p < 0.5$, Figure 4-4 and Table 4-4). During the 16-week 3°C incubation a significantly lower

NH	Eggs Laid at 3°C																						Eggs Laid at 0°C																	
	Total		wk0 20°C Incubation					3° Incubation											wk16 20°C Incubation					Total		wk0 20°C Incubation					3°C Incubation									wk16 20°C Incubation				
	#		#	d3		d6		wk1		wk2		wk4		wk8		wk12		wk16		#	d3		d6		#		#	d3		d6		wk1		wk2		wk4		wk8		wk12		wk16		#	d3		d6	
♀	E	N	E	E	N	E	N	E	N	E	N	E	N	E	N	E	N	E	N	E	E	N	E	N	E	N	E	N	E	N	E	N	E	N	E	N	E	N	E	N	E	N	E	E	N	E	N
1	20	3	10	3	7	0	3	9	1	7	1	6	1	5	1	5	0	5	0	5	3	2	0	3	16	0	8	2	6	0	2	7	1	6	1	2	4	2	0	2	0	2	0	2	2	0	0	2
2	15	1	7	5	0	0	4	8	0	7	0	6	0	6	0	4	1	4	0	4	2	2	0	2	5	1	2	0	2	0	0	3	0	2	1	1	0	1	0	1	0	1	0	1	0	1	0	0
3	20	8	10	2	6	0	2	10	0	5	0	5	0	5	0	5	0	5	0	5	4	1	0	3	18	2	9	4	5	0	4	8	0	7	0	7	0	7	0	7	0	7	0	7	3	3	0	3
4	7	3	3	1	2	0	1	4	0	2	0	2	0	1	1	0	0	0	0	0	0	0	0	0	13	1	6	1	5	0	1	7	0	7	0	7	0	6	1	6	0	6	0	6	2	3	0	2
5	1	2	0	0	0	0	0	1	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	7	0	3	1	1	0	1	4	0	4	0	4	0	3	1	3	0	3	0	3	3	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	7	13	3	0	3	0	0	3	1	3	0	2	0	2	0	2	0	2	0	2	1	1	0	0	2	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	6	6	3	0	3	0	0	3	0	3	0	2	0	2	0	2	0	2	0	2	0	2	0	0	3	2	1	0	1	0	0	2	0	2	0	2	0	2	0	2	0	2	0	2	2	0	0	1
9	22	5	11	5	6	0	5	11	0	11	0	10	0	7	2	7	0	6	0	6	2	4	0	1	11	1	5	0	5	0	0	4	2	2	0	2	0	2	0	2	0	2	0	2	0	2	0	0
10	2	2	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	1	1	1	0	1	0	1	0	1	0	1	0	1	0	1	0	0
11	2	3	1	0	1	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	0	0	1	3	0	1	0	1	0	0	2	0	2	0	2	0	1	1	1	0	1	0	1	0	1	0	0
12	4	4	2	2	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2	1	0	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	23	17	10	4	6	0	4	11	2	9	0	3	1	2	0	2	0	2	0	2	0	1	0	0	17	13	8	0	8	0	0	5	4	4	1	2	2	2	0	2	0	2	0	2	1	1	0	1
14	18	3	9	2	7	0	2	9	0	8	0	5	2	5	0	5	0	5	0	5	2	3	0	1	8	1	4	0	3	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	11	17	5	0	5	0	0	6	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
16	12	5	6	0	6	0	0	6	0	6	0	5	0	4	1	4	0	4	0	4	3	1	0	2	6	0	3	0	2	0	0	3	0	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0
17	10	4	5	0	5	0	0	5	0	3	0	2	0	2	0	2	0	2	0	2	0	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18	20	12	10	2	8	0	1	10	0	6	2	5	1	5	0	4	1	4	0	4	2	2	0	2	2	3	1	0	1	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	0
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	5	14	2	0	2	0	0	2	1	2	0	2	0	2	0	2	0	2	0	2	1	1	0	1	2	3	1	0	1	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	1	0	0	1
21	7	4	3	2	1	0	2	3	1	3	0	3	0	3	0	3	0	3	0	3	0	3	0	0	5	0	2	0	2	0	0	3	0	3	0	3	0	2	1	2	0	2	0	2	0	2	0	0
22	2	2	1	0	1	0	0	1	0	1	0	1	0	1	0	1	0	1	0	2	1	0	0	1	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	7	2	3	1	2	0	1	3	0	3	0	2	0	2	0	2	0	2	0	2	1	1	0	1	8	1	4	0	1	0	0	2	2	1	1	1	0	1	0	1	0	1	0	1	1	0	0	1

Table 4-1: Experimental results from NH cultures. ♀ = female in egg laying chamber, E = eggs, N = nauplii, Total is the total number of eggs and nauplii counted in chamber after 1-week egg laying incubation, d = day, wk = week, # is the number of eggs at the beginning of the 20°C incubations, all numbers except numbers identifying females are counts.

NC	Eggs Laid at 3°C																						Eggs Laid at 0°C																					
	Total		wk0 20°C Incubation					3° Incubation											wk16 20°C Incubation					Total		wk0 20°C Incubation					3°C Incubation											wk16 20°C Incubation				
	#		#	d3		d6		wk1		wk2		wk4		wk8		wk12		wk16		#	d3		d6		#		#	d3		d6		wk1		wk2		wk4		wk8		wk12		wk16		#	d3		d6	
♀	E	N	E	E	N	E	N	E	N	E	N	E	N	E	N	E	N	E	N	E	E	N	E	N	E	N	E	E	N	E	N	E	N	E	N	E	N	E	N	E	N	E	N	E	E	N	E	N
1	9	2	4	2	2	0	2	3	2	3	0	3	0	3	0	3	0	3	0	3	2	1	0	1	16	2	8	0	0	0	0	7	0	6	0	6	0	6	0	6	0	6	0	6	4	0	0	0
2	9	2	4	0	4	0	0	5	0	5	0	5	0	4	1	4	0	4	0	4	2	2	0	1	3	0	1	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	5	6	2	0	2	0	0	2	1	2	0	2	0	2	0	2	0	2	0	2	0	2	0	0	3	0	1	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	9	3	4	1	3	0	1	5	0	5	0	4	1	3	1	3	0	3	0	3	0	3	0	0	2	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	5	3	2	1	1	0	1	2	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	1	2	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	6	1	3	1	1	0	1	3	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	8	0	4	2	2	0	2	3	1	3	0	2	1	2	0	2	0	2	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	6	3	3	1	1	0	1	3	0	3	0	3	0	3	0	3	0	3	0	3	1	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	2	6	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	1	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	3	3	1	1	0	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18	1	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	5	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	4	0	2	1	1	0	1	2	0	2	0	2	0	2	0	2	0	2	0	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4-2: Experimental results from NC cultures. ♀ = female in egg laying chamber, E = eggs, N = nauplii, Total is the total number of eggs and nauplii counted in chamber after 1 week egg laying incubation, d = day, wk = week, # is the number of eggs at the beginning of the 20°C incubations, all numbers except numbers identifying females are counts.

Culture	Temp	Total # Eggs	Eggs/♀	Upper 95% CI	Lower 95% CI	% ♀ Laid Eggs	% ♀ Produce Nauplii	# Eggs wk0 20°C Incubation	# Eggs 3° Incubation	# Eggs wk16 20°C Incubation
NH	3°C	358	12.71	17.78	8.47	91.7	91.7	108	250	54
	0°C	166	5.58	8.29	3.37	91.7	87.5	58	108	29
NC	3°C	110	3.60	5.51	2.05	79.2	79.2	30	80	19
	0°C	37	0.71	1.73	0.00	20.8	4.2	16	21	6

Table 4-3: Total number of eggs, number of eggs per female, and upper and lower confidence intervals, percent of females that laid eggs, and percent of females that produced nauplii for eggs laid over 1 week from NH and NC cultures at 3°C and 0°C (mean and upper and lower confidence intervals calculated after square root transformation of data). Also shown are the number of eggs used for 20°C incubation at week 0, 3°C 16 week incubation, and 20°C incubation at week 16.

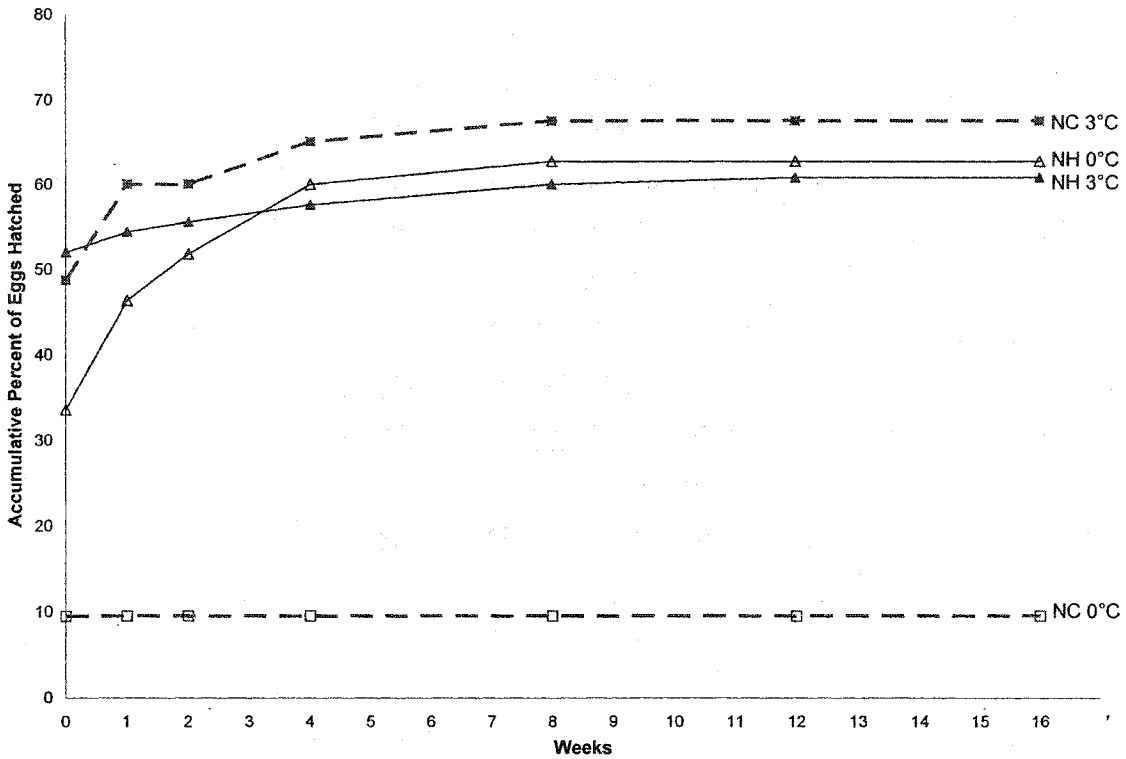

Figure 4-3: Accumulative percent of eggs hatched during the 16-week incubation at 3°C from NH and NC laid at 3°C or 0°C.

percent of eggs hatched than during either 20°C incubation ($p < 0.01$ for all comparisons, Table 4-4). There was no significant difference for eggs laid at 3°C and 0°C in the percent of eggs hatched during the 16-week incubation at 3°C ($0.5 > p > 0.2$). The overall percentage of NH eggs laid at 3°C that hatched during the 16-week incubation at 3°C was 60.8%, ranging from 22.2% to 88.2% (Figure 4-3). Among the eggs that hatched, 85.5% hatched during the 1-week egg-laying period and 89.5% had hatched by the end of the first week of the 3°C incubation (Figure 4-3). The overall percent of NH eggs laid at 0°C that hatched during the 16 week 3°C incubation was 62.7% with a range from 0% to 100%. The highest and lowest values were from samples with 3 or fewer eggs. Among the eggs that hatched, 53.6% hatched during the 1-week egg-laying period,

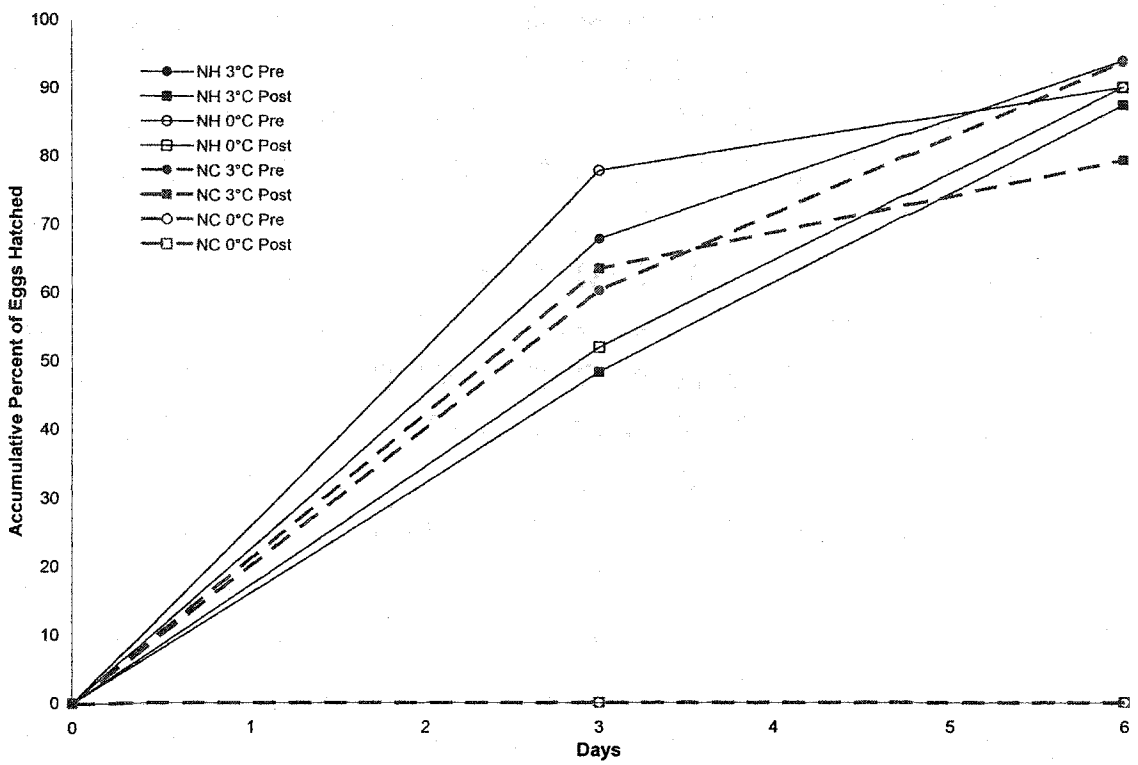

Figure 4-4: Accumulative percent of eggs hatched during the 6-day incubation at 20° before (pre) and after (post) the 16-week incubation at 3°C from NH and NC laid at 3°C and 0°C.

and 73.9% hatched within the first week of the 3°C incubation (Figure 4-3). For all of the 20°C incubations, all eggs hatched or disintegrated.

For the NC eggs laid at 3°C, there was no significant difference between the percent of eggs hatched during the 20°C incubations at week 0 and week 16 ($p > 0.1$ for both experiments, Figure 4-4, Table 4-4). There was a significantly lower percent of eggs hatched during the 16-week 3°C incubation than during the week 0 20°C incubation ($0.025 > p > 001$); and no significant difference in the percent of eggs hatched during the 16-week 3°C incubation and the week 16 20°C incubation ($0.2 > p > 0.1$, Table 4-4). No comparisons were made for the NC eggs laid at 0°C, because only one female produced viable eggs. The overall percent of NC eggs laid at 3°C hatching during the 16-week 3°C

Comparison	Test	p	Multiple Comparisons (p)
NH 3°C (pre, during, post)	Friedman's ANOVA	<0.0001*	pre = post (p > 0.50) pre ≠ during (p < 0.001*) post ≠ during (0.005> p >0.001*)
NH 0°C (pre, during, post)	Friedman's ANOVA	0.0009*	pre = post (p > 0.50) pre ≠ during (0.01> p >0.005*) post ≠ during (0.01> p >0.005*)
NC 3°C (pre, during, post)	Friedman's ANOVA	0.0037*	pre = post (p > 0.50) pre ≠ during (0.025> p >0.01*) post = during (0.2> p >0.1)
NH 3°C during and NH 0°C during	Wilcoxon paired-sample test	0.5 > p > 0.2	
NH 3°C and NC 3°C during	Mann-Whitney test	<0.0001*	
NH 3°C pre and NC 3°C pre	Mann-Whitney test	1.0000	
NH 3°C post and NC 3°C post	Mann-Whitney test	0.0949	

Table 4-4: Comparisons of the percent of eggs hatched between different groups. 3° and 0°C are the egg laying temperature. Pre represents the percent of eggs hatched in the initial move of eggs from 3° or 0°C to 20°C at week 0; during represents the percent of eggs hatched during the 16-week 3°C incubation; and post represents the percent of eggs hatched in the final move of eggs from 3°C to 20°C at week 16. All comparisons are based on the accumulative percent of eggs hatched in each chamber over the entire incubation period. All test are nonparametric rank sum test, multiple comparisons used a nonparametric Tukey-type test with rank sums. For blocked (Friedman's ANOVA) and paired-sample test (Wilcoxon) only samples with complete data were used. * indicates significant difference.

incubation was 67.5% with a range from 0% to 100% (Figure 4-3). Among the eggs that hatched, 72.2% hatched during the 1-week egg-laying period; 88.9% had hatched by the end of the first week of the 3°C incubation (Figure 4-3). For all of the 20°C incubations, all eggs hatched or disintegrated.

Comparisons between the of NH and NC eggs laid at 3°C showed no significant difference in the percentages of eggs hatched during either the week 0 (p = 1) or week 16 (p = 0.0949) 20°C incubations (Table 4-4). A significantly lower percentage of NH eggs hatched during the 16 week 3°C (Table 4-4).

DISCUSSION

It is assumed from studies on neighboring populations that *Acartia tonsa* in Great Bay, NH over-winter as dormant eggs, while *A. tonsa* in Beaufort Inlet, NC remain in the water column year round. The most notable differences observed between the NH and NC cultures were a higher percentage of NC eggs hatching during the 3°C incubation, and the absence of viable eggs produced by NC females at 0°C. These findings correlate well with the environmental histories of the cultures' source populations. It is impossible to say whether these results have a causative basis. Interestingly, similarities between the cultures included a high percentage of egg hatching at 20°C (both before and after the 16 week incubation at 3°C) and almost no eggs hatching after the eighth week at 3°C. These similarities may provide insight into the mechanisms controlling egg dormancy in *A. tonsa*.

By definition, diapause eggs are predisposed to enter a dormant state in response to an environmental cue and usually require the egg to undergo an incubation period prior to hatching (Danks 1987). This experiment showed that *A. tonsa* eggs laid at low temperatures (0° - 3°C) with a short photoperiod (6:18hr light:dark) can hatch immediately if brought to 20°C or can remain dormant if left at 3°C. Thus, *A. tonsa* lay quiescent rather than diapause eggs under these culture conditions. Similar results have been observed in other studies (Zillioux and Gonzalez 1972; Chen and Marcus 1997). The current study differs from these by showing that no incubation period was necessary to break dormancy in *A. tonsa* eggs. Another difference is that Zillioux and Gonzalez (1972), reported that no eggs laid at 5°C hatched after incubations of 30 to 135 days at

5°C. In this study, 58% to 68% of the eggs laid at either 0°C or 3°C hatched at 3°C, most within the first 8 weeks. This difference may result from the acclimation of females to 10°C prior to egg laying by Zillioux and Gonzalez (1972), while females were acclimated to 3°C in this study. Tester (1985) has shown that parental acclimation temperature does effect egg development time. This, and other unknown culturing differences (e.g., photoperiod is not recorded in all studies) could explain the differences in the results.

This study, together with previous studies, indicates that the response to low temperature by *A. tonsa* eggs involves active control of metabolic processes. This can be shown by Belehradek's equation (Beleharadek 1926; McLaren et al. 1969) which predicts temperature-dependent egg development times, assuming no physiological acclimation. McLaren et al (1969) calculated the constants for Belehradek's equation for *A. tonsa* from Narragansett Bay RI, and predicted a development times for *A. tonsa* eggs of 337 days at 3°C, and 45 days at 5°C. In this study, eggs that remained dormant for 16 weeks (112 days) at 3°C could have been developing at a non-adjusted metabolic rate. However, the eggs that hatched within a few weeks at 3°C probably acclimated to the low temperature by increasing their temperature-dependent metabolic rates. In contrast, Zillioux and Gonzalez (1972) observed eggs that remained dormant at 5°C for 135 days, well beyond the predicted development time of 45 days. Thus, the metabolic rate of these eggs must have been slowed down or stopped in response to the low temperature.

One possible mechanism to explain these results is a temperature sensitive metabolic switch, which responds to low temperature by either increasing the egg's metabolic rate (so it can develop) or reducing it (sending it into a dormant state). Among eggs from the

same female, it was common for some fraction to hatch immediately, while the remaining fraction remained dormant. This suggest that development time is determined by the eggs genotype rather than maternal effects. However, it is not possible to determine from this data whether such a switch may be controlled genetically or environmentally. It should be noted that, if the control is heritable, then there might have been artificial selection in this study in favor of eggs that would develop at low temperature rather than go dormant, since the parental generations were cultured from eggs laid and developed at 5°C.

These results only show that *A. tonsa* failed to produce diapause eggs under the culture conditions of this study. While photoperiod and/or temperature seem to be the major cues to induce females to lay diapause eggs in copepods (Marcus 1982a; Hairston and Kearns 1995; Avery 1999; Chinnery and Williams 2003), it is possible that *A. tonsa* relies on a different cue, or the cue was not presented appropriate in this study (i.e., the cue might have been a shortening of day length or a reduction in temperature, both of these were held constant in this study). More investigations are needed before a conclusion can be reached about *A. tonsa's* ability to lay diapause eggs in the wild. Indeed the only definitive experiment might be to conduct *in situ* experiments on *A. tonsa* directly in the estuaries.

Independent of the control mechanisms, if *A. tonsa* do not lay diapause eggs, then the reduced evolutionary rate hypothesized to occur in some copepods because of the existence of an egg bank (Hairston and DeStasil 1988) is probably not occurring in *A. tonsa*. In fact, the high levels of genetic diversity and significant genetic divergence

among geographic populations suggest that rates of evolution are rapid for *A. tonsa* (see Chapter 2).

CONCLUSIONS

Acartia tonsa from NH and NC are capable of laying quiescent eggs in laboratory cultures at low temperature with a short photoperiod. Experiments exploring rates of egg laying, development, and hatching in response to different temperatures indicate that the dormant eggs produced in culture are not diapause eggs. If the results hold for wild populations then it is unlikely that *A. tonsa* eggs' are capable of remaining in the sediment over multiple seasons and therefore unlikely to form an egg bank. However, the results of this study are not directly applicable to wild populations, which may lay diapause eggs and create an egg bank. The experimental results are consistent with a temperature sensitive metabolic switch controlling whether an egg enters a quiescent state or continues to develop at low temperatures.

CHAPTER V

MULTIPLEXED SPECIES-SPECIFIC PCR PROTOCOL TO DISCRIMINATE FOUR N. ATLANTIC *CALANUS* SPECIES, WITH A mtCOI GENE TREE FOR TEN *CALANUS* SPECIES

ABSTRACT

Accurate species identification is the cornerstone of any ecological study - yet this fundamental step is not always possible for marine zooplankton. Routine species identification, especially of juvenile and larval stages, is difficult for *Calanus* species (Copepoda; Calanoida) in the N. Atlantic Ocean, where two or three species may co-occur. A rapid, simple, and inexpensive molecularly-based protocol to identify individual copepods of any life stage has been developed. This protocol will routinely identify four *Calanus* species in the N. Atlantic, allowing opportunity for accurate understanding of the role of each species in coastal and open ocean ecosystems. The DNA sequence of a 633 base-pair region of the mitochondrial cytochrome oxidase I (mtCOI) was determined for ten *Calanus* species: *C. australis* (Brodsky, 1959), *C. chilensis* (Brodsky, 1959), *C. finmarchicus* (Gunnerus 1770), *C. glacialis* (Jaschnov 1955), *C. helgolandicus* (Claus 1863), *C. hyperboreus* (Kroyer 1838), *C. marshallae* (Frost 1974), *C. pacificus* (Brodsky 1948), *C. simillimus* (Giesbrecht, 1902), and *C. sinicus* (Brodsky 1965). MtCOI sequences were used to design species-specific oligonucleotide primers for *C. finmarchicus, C. glacialis, C. helgolandicus*, and *C.*

hyperboreus and optimize a competitive, multiplexed, species-specific PCR (SS-PCR) protocol to discriminate the four species. This corrects and improves a previously published protocol for three *Calanus* species (Bucklin et al. 1999, Hydrobiologia 401:239), unambiguously identifying individual copepods and copepodites from diverse geographic regions of the four species' ranges. In order to further examine the pattern of mtCOI evolution within *Calanus* (an important consideration for molecular systematic characters), consensus mtCOI sequences were used to reconstruct phylogenetic relationships among the ten species; the mtCOI gene tree agreed with morphological and molecular (based on mt 16S rRNA) phylogenies, except that the affiliation of *C. sinicus* could not be resolved.

INTRODUCTION

Four species of *Calanus* (*C. finmarchicus, C. helgolandicus, C. glacialis*, and *C. hyperboreus*) may co-occur throughout the N. Atlantic Ocean (Fleminger and Hulsemann 1977; Bucklin et al. 2000a). Larval and juvenile stages of the abundant and ubiquitous species, *Calanus finmarchicus*, are not readily discriminated from those of a sibling species, *C. glacialis,* and a non-sibling species, *C. helgolandicus*, which have overlapping geographic ranges (Frost 1974). The ecological importance of *Calanus* species (Copepoda; Calanoida) in coastal and oceanic ecosystems makes routine species' identification, especially for juvenile and larval stages, both problematical and important (Fleminger and Hulsemann 1977, 1987; Frost 1971, 1974). Species' identification based

on size and collection location - a frequent tactic for biological oceanographers - is not sufficient in the N. Atlantic, where three or four *Calanus* species may co-occur (Bucklin et al. 1999, 2000a) and geographic variation in environmental conditions may affect individual size (Frost 1971, 1974). Discrimination of all life stages of these species is essential to understanding recruitment and other population dynamic processes of the N. Atlantic zooplankton assemblage.

As a group, calanoid copepods (Crustacea; Copepoda; Calanoida) include numerous groups of sibling species; they are among the most species-rich holoplanktonic invertebrates in the oceans (McGowan 1971). The subtle morphological changes accompanying reproductive isolation of the species have resulted in difficulties in identification (see e.g., Fleminger and Hulsemann, 1977; Frost 1989). Recent molecular assessment of several genera of marine calanoid copepods has determined that congeneric species, despite their morphological similarity, exhibit significant divergence in the sequences of mitochondrial genes (Bucklin et al. 1992, 1995, 1998a, 1998b, 1999, unpubl.). Molecular systematic assessment of calanoid copepods may thus help define species boundaries, reveal cryptic species, identify morphologically-indistinguishable taxa (for individuals, pooled samples, and gut contents), and reconstruct evolutionary patterns within speciose groups by molecular phylogenetic analysis.

Useful molecular approaches include: comparing a partial DNA sequence for a selected gene portion with a DNA "type" sequence (i.e., a DNA sequence of an individual from the type locality serving as a taxonomic reference); population genetic examination to

understand the systematic significance of geographic variation; design of rapid protocols, such as species-specific PCR, for species' identification.

The evolutionary history of *Calanus* species is unclear, in part because they have no fossil representatives. Frost (1974) and Fleminger and Hulsemann (1977) hypothesized that *Calanus* species experienced a recent radiation - possibly over tens of thousands to a few millions of years - but many questions remain about the evolutionary histories of the genus and family (Bradford and Jillett 1974; Bradford 1988). Current understanding of the evolutionary relationships among the 14 species of *Calanus* is based on morphological and morphometric analysis (Frost 1974; Fleminger and Hulsemann 1977, 1987), and molecular phylogenetic analysis of mt 16S rRNA sequence variation (Bucklin et al. 1992, 1995). These studies agree that there are two sibling species groups: the *finmarchicus* group (comprising the polar and boreal species of the northern hemisphere, *C. finmarchicus*, *C. glacialis*, and *C. marshallae*) and the *helgolandicus* group (comprising species found in mid-latitudes of both hemispheres, *C. helgolandicus*, *C. agulhensis*, *C. australis*, *C. chilensis*, *C. euxinus*, *C. orientalis*, *C. pacificus*, and *C. sinicus*; Fleminger and Hulsemann 1987, Hulsemann 1991; DeDecker et al. 1991). Two species, *C. hyperboreus* and *C. simillimus*, are distinct from either group (Frost 1971, 1974).

Several protocols based on molecular genetic characters have been successfully applied to marine zooplankton (see review by Bucklin 2000). Short (20 to 50 base pairs) regions of DNA sequence diagnostic of a particular species may be used to design species-specific oligonucleotide primers and probes and restriction enzyme digestion assays (see

Griffin and Griffin 1994; Bucklin 2000). Oligonucleotide probes have been used to identify phytoplankton cells (DeLong et al. 1989; Scholin and Anderson 1996) and marine invertebrate larvae (Olson et al. 1991; Banks et al. 1993; Medeiros-Bergen et al. 1995; Bell and Grassle 1998). Competitive species-specific PCR (see Gibbs et al. 1989) has been used for a variety of marine organisms (Dixon et al. 1995; Fell 1995; Bucklin et al. 1998a, 2000b). For *Calanus* species in particular, Lindeque et al. (1999) designed a three-step protocol based on PCR amplification of mitochondrial 16S rRNA, restriction enzyme digestions, and agarose gel electrophoresis to distinguish *C. finmarchicus, C. helgolandicus*, *C. hyperboreus*, and *C. glacialis*.

We selected allele-specific amplification by the polymerase chain reaction (PCR; see Charlieu 1994) for discrimination of *Calanus* species. Because the alleles are selected to be diagnostic of each species, we have termed this species-specific PCR (SS-PCR). Typically, SS-PCR reactions are "multiplexed" (i.e., carried out simultaneously and competitively in a single tube; Gibbs et al. 1989). Thus a single SS-PCR reaction may allow discrimination among multiple species, through the use of an "anchor" oligonucleotide primer (i.e., a sequence portion shared by all species) and multiple, competing species-specific primers (i.e., each sequence portion is unique to one species). The results are determined by sizing the PCR products by agarose gel electrophoresis.

Using mitochondrial cytochrome oxidase I (mtCOI) sequence variation, Bucklin et al. (1999) designed a competitive, multiplexed SS-PCR protocol to discriminate three N. Atlantic *Calanus* species, here modified to discriminate four N. Atlantic *Calanus* species. The current study corrects an error (*C. glacialis* was previously misidentified by Bucklin

et al. 1999); improves the protocol (the *C. finmarchicus* primer is now based on the coding gene sequence, rather than a putative pseudogene); and expands the analysis to include four *Calanus* species.

Our selection of mtCOI as a molecular systematic character was founded on examination of intra- and interspecific variation in the DNA sequence of 10 *Calanus* species. These results are summarized in a mtCOI gene tree representing both the evolutionary relationships among *Calanus* species and the evolution of the gene in the genus.

MATERIALS AND METHODS

Collection, preservation, and identification of *Calanus* species samples:

Samples of *Calanus* species were collected from diverse regions of the world oceans, including the N. and S. Atlantic, N. and S. Pacific, and Arctic Oceans (Table 5-1). The species were usually identified by the collectors, who are copepod taxonomic experts; western N. Atlantic samples were collected and identified by the authors (Table 5-1). All samples were preserved in 95% ethyl alcohol as described by Bucklin (2000).

DNA sequence determination for *Calanus* species:

Individual copepods were prepared for molecular analysis by boiling in distilled water for 10 to 15 min to evaporate the alcohol. The molecular analyses also used DNA purified from pooled individuals by phenol extraction and ethanol precipitation (Bucklin 2000).

Species	Sampling Region	Location	Date	Collector
C. finmarchicus	Georges Bank, NW Atlantic	41o28'N; 66o47'W	21-Jun-98	A. Bucklin
C. glacialis	Arctic Ocean	78o N; 160o W	8-Jul-98	C. Ashjian
C. marshallae	Puget Sound, NE Pacific	47o41'N; 122o50'W	22-May-96	B.W. Frost
C. helgolandicus	Northeast Atlantic (NE)	49o30'N; 4o25'W	17-Jul-91	R.P. Harris
	North Atlantic (NA)	38o43'N; 42o11'W	24-Apr-93	A. Bucklin
	Adriatic Sea (AD)	42o45'N; 17o30'E	21-Dec-95	A. Benovie
C. pacificus				
C. p. californicus	Puget Sound, NE Pacific	47o46'N; 122o50'W	22-May-96	B.W. Frost
C. p. oceanicus	Subarctic Gyre, N. Pacific	50o00'N; 145o00'W	27-Aug-96	B.W. Frost
C. sinicus	Inland Sea of Japan	34o30'N; 132o30'E	23-Jan-93	S.-I. Uye
C. chilensis	Mejillones Bay, Chile	23o S; 70o N	19-May-95	I. McLaren
C. australis	S. Pacific Ocean	44o 00' S; 178o 30' E	25-Oct-97	J. Bradford Grieve
C. simillimus	S. Pacific Ocean	46o 40' S; 178o 29' E	20-Oct-97	J. Bradford Grieve
C. hyperboreus	Gulf of St. Lawrence, NW Atlantic	48o40'N; 68o35'W	17-Aug-92	J. Runge

Table 5-1: Collection information for *Calanus* species used for molecular phylogenetic analysis.

For *C. finmarchicus*, complementary DNA (cDNA) was synthesized from purified mRNA using the Simple Nucleic Acid Preparation (S.N.A.P.) Total RNA Isolation Kit (Invitrogen, Inc., Carlsbad, CA). The use of cDNA for *C. finmarchicus* was necessitated by the presence of a non-coding pseudogene (not found in other species) which was preferentially amplified by our PCR primers.

A ~700 bp region of mtCOI was amplified using consensus primers made according to published sequences (Folmer et al. 1994):

LCO-1490 5' - GGT CAA CAA ATC ATA AAG ATA TTG G - 3'

HCO-2198 5' - TAA ACT TCA GGG TGA CCA AAA AAT CA - 3'.

The mtCOI primers are named based on position numbers of the *Drosophila yakuba* sequence (Clary and Wolstenholme 1985). Although amplification protocols for each species varied slightly, they were all similar to a standard protocol: dissociation at 94° C (1 min), annealing at 37° C (2 min), and extension at 72° C (3 min) for 40 cycles.

Annealing temperatures of 39° or 45° C were also used. For confirmation (since a putative COI pseudogene was observed for some species; Bucklin et al. 1999), PCR amplification was also done using the primers, LCO-1703 and LCO-1719, in place of LCO-1490 for *C. finmarchicus, C. glacialis, C. marshallae, C. helgolandicus*, and *C. chilensis*. The primers were designed from mtCOI sequences obtained as part of this study. The primer sequences are:

LCO-1703 5' - CTA TTTT GAT TGG AGG ATT TGG - 3'

LCO-1719 5' - GGA TTT GGT AAC TGA TTA GTG CC - 3'

The DNA sequencing was done by direct sequencing of PCR amplification products, using the LCO-1490 primer, according to published protocols (Bucklin 2000). PCR products obtained using LCO-1703 or LCO-1719 used that primer for sequencing. Sequencing was carried out on an American Biotechnology, Inc., Model 373, automated DNA sequencer.

The DNA sequence of a 633 base-pair (bp) region of mtCOI gene for each species was determined by PCR from individual copepods (sample sizes indicated by N) or by purification of genomic DNA from multiple individuals pooled prior to analysis (except that cDNA was purified for *C. finmarchicus* to avoid amplification of the non-coding COI pseudogene). Sample sizes or pool sizes for each species were: *C. australis* (N = 3), *C. chilensis* (N = 2 and pool of 25), *Calanus finmarchicus* (pool of 6), *C. glacialis* (N = 6), *C. helgolandicus* from the Adriatic Sea (pool of 30), *C. helgolandicus* from the central N. Atlantic (pool of 12), *C. helgolandicus* from the NE Atlantic (pools of 13 and 20), *C.*

hyperboreus (N = 2 and pool of 2); *C. marshallae* (N = 3 and pool of 20), *C. pacificus californicus* (pools of 14 and 26) and *C. p. oceanicus* (N = 7 and pool of 20), *C. simillimus* (N = 2), and *C. sinicus* (N = 1 and pool of 16). For each species, the consensus sequence of all sequences obtained was used for subsequent analyses.

Species-specific PCR protocol design:

Species-specific oligonucleotide primers were designed from the consensus mtCOI sequences by identifying regions of 20 - 30 bp that were conserved within each species (based on our limited examination of intraspecific variation) and differed from all other *Calanus* species by ~25% of the bases. Primer sequences were evaluated for suitable base composition, temperature of dissociation, and self-compatibility using the software programs OLIGO (Rychlik 1992) and *Amplify* (Engels 1992). Multiplexing of the PCR reactions (i.e., simultaneous and competitive PCR using multiple species-specific primers and one common primer; Gibbs et al. 1989) and discrimination of the different-sized PCR products by agarose gel electrophoresis were made possible by selecting primers at different sites along the 634 bp sequence. Thus, the lengths of the amplified products for each species differed and were, in order of increasing size: 117 bp for *C. finmarchicus*; 288 bp for *C. helgolandicus*; 428 bp for *C. glacialis*; and 606 for *C. hyperboreus*.

The amplification protocol for the multiplexed SS-PCR reaction was: 94°C (0.5 min); 47°C (0.5 min); 72°C (1 min); for 35 cycles (see Appendix 1) All sets of SS-PCRs included positive controls for each species (i.e., genomic DNA purified from *Calanus* species identified by an expert taxonomist). The amplified products of each species were discriminated by electrophoresing the products of the multiplexed, competitive reaction

on a 2.0% agarose gel. The specificity and reliability of the multiplexed PCR reactions were confirmed by examining individuals collected in different regions of the Arctic and N. Atlantic, including Gulf of Maine, Norwegian Sea and several Norwegian fjords.

Phylogenetic reconstruction for *Calanus* species:

Phylogenetic relationships among the 10 *Calanus* species were assessed using the consensus DNA sequences and the algorithms of the Molecular Evolutionary Genetics Analysis (MEGA), Ver. 1.0, software package (Kumar et al. 1993). The definitive analysis, yielding the highest statistical significance overall, used Neighbor Joining (Saitou and Nei 1987), with Tamura-Nei distances (Tamura and Nei 1993) and an empirically-derived alpha parameter of 2.0. The trees were bootstrapped 1000 times.

RESULTS

MtCOI sequence variation:

The consensus DNA sequence for a 633 bp region of mtCOI was determined for ten species of *Calanus* (see Appendix 2): *C. australis* (GenBank Accession No. AF332766); *C. chilensis* (GenBank Accession No. AF332765); *C. finmarchicus* (GenBank Accession No. AF332767); *C. glacialis* (GenBank Accession No. AF333039); three populations of *C. helgolandicus* (GenBank Accession Nos. AF332760, AF332761, and AF332762); *C. hyperboreus* (GenBank Accession No. AF332770), *C. marshallae* (GenBank Accession No. AF332768); two subspecies of *C. pacificus, C. p. californicus* (GenBank Accession

	C.mar	*C.sin*	*C.hyp*	*C.sim*	*C.h-D*	*C.h-A*	*C.h-E*	*C.p.c*	*C.p.o.*	*C.chi*	*C.aus*	*C.fin*
C.glacialis	0.07	0.21	0.23	0.26	0.25	0.25	0.25	0.22	0.23	0.21	0.24	0.23
C.marshallae		0.21	0.22	0.23	0.22	0.22	0.22	0.22	0.23	0.21	0.23	0.19
C.sinicus			0.21	0.21	0.20	0.20	0.20	0.22	0.22	0.22	0.21	0.22
C.hyperboreus				0.19	0.21	0.20	0.20	0.22	0.22	0.22	0.20	0.21
C.simillimus					0.21	0.22	0.21	0.23	0.23	0.21	0.23	0.23
C.helgolandicus-AD						*0.01*	*0.01*	0.20	0.21	0.20	0.19	0.21
C.helgolandicus-NA							*0.01*	0.21	0.21	0.20	0.19	0.21
C.helgolandicus-NE								0.20	0.21	0.20	0.19	0.21
C.p. californicus									*0.03*	0.11	0.17	0.23
C.p. oceanicus										0.12	0.17	0.23
C.chilensis											0.16	0.19
C.australis												0.20

Table 5-2: Proportion of nucleotide differences between *Calanus* species based on a 633 base-pair region of mtCOI. Collection locations are in Table 5-1. Data in italics represents comparisons between populations of the same species. Abbrevations for *C. helgolandicus* populations are: Adriatic Sea (AD), North Atlantic (NA), and Northeast Atlantic (NE)

No. AF332763) and *C. p. oceanicus* (GenBank Accession No. AF332764); *C. simillimus* (GenBank Accession No. AF332771); and *C. sinicus* (GenBank Accession No. AF332769). The nucleotide sequences differed by 7 to 25% between *Calanus* species, by about 3% between *C. pacificus* subspecies, and by < 1% between geographic populations of *C. helgolandicus* (Table 5-2).

Discrimination of *Calanus* species by species-specific PCR:

The species-specific primers were used with the common primer COI-2128R (5' - GTG CTG RTA TAA AAT AGG - 3'). The sequences and positions of the species-specific primers were:

C. finmarchicus (COI-2011) 5'- YTC ATC ACT GCT GTC CTC -3'

C. glacialis (COI-1700) 5'- TTA TGT TGG GTG CGG CGG AC -3'

C. helgolandicus (COI-1840) 5'- CTA TCC AGA AAT GTA GCC -3'

C. hyperboreus (COI-1522) 5'- TCA GGA ATG ATC GGA ACC -3'

The degenerate (i.e., based on amino acid sequences) primers, COI-2128R and COI-2011, were synthesized as equimolar mixtures of all possible sequences. The primers differed somewhat in their dissociation or melting temperatures (T_M, for methods see Breslauer et al. 1986; http://www.nwu.edu/biotools/oligocalc.html). T_M ranged from 41 - 43° C for COI-2128R and from 48 - 50° C for COI-2011. T_M was 56° C for COI-1700, 46° C for COI-1840, and 46° C for COI-1522.

The competitive, multiplexed SS-PCR reaction was designed to allow species' identification with two steps - PCR amplification and gel electrophoresis - by the placement of the competing species-specific primers at different sites along the mtCOI sequence. The SS-PCR reaction yielded only one product band (in addition to primer-dimer, which appeared as a low molecular-weight band on the gel) in nearly all cases (Figure 5-1). An exception was that amplification of *C. helgolandicus* collected from Norwegian fjords resulted in the correct-sized product band and a second artefactual band, which differed in size from any product of the positive control reactions.

The feasibility of using the SS-PCR reaction to identify individuals of the four *Calanus* species was demonstrated by assaying copepods sorted from zooplankton collections from several regions in the N. Atlantic and one in the Arctic Ocean. The results were unambiguous. There were several instances of >10% PCR failure (i.e., reactions yielding no product; Table 5-3), which were considered to be a result of poor sample preservation.

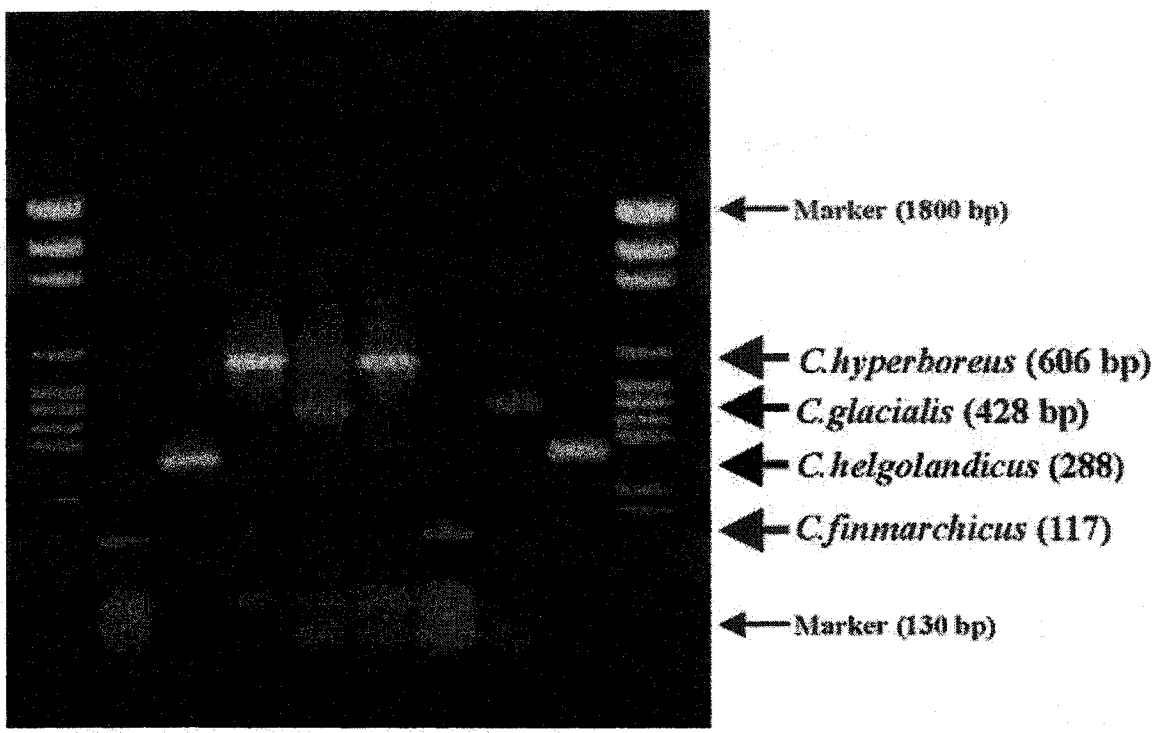

Figure 5-1: Photograph of the species-specific amplification products for each of the four *Calanus* species resulting from the multiplexed SS-PCR protocol, with molecular size standards (first and last lanes of the gel). Amplification products differ in size, resulting in different positions of the bands in agarose gel electrophoresis.

These experiments confirmed the predominance of *C. hyperboreus* in a sample from the Arctic Ocean; the presence of *C. finmarchicus* and *C. glacialis* in a sample from the Gulf of Maine; the predominance of *C. finmarchicus* in the Norwegian Sea; and a mixture of all four *Calanus* species in fjords of western Norway (Table 5-3).

Phylogenetic reconstruction:

The consensus mtCOI nucleotide sequences were used to reconstruct the phylogenetic history of the genus, and especially to determine the pattern of speciation within sibling species groups. With one exception, the mtCOI gene tree resolved species relationships with statistically significant bootstrap values and clear resolution of all species, subspecies, and populations. The exceptions were *C. finmarchicus* and *C. sinicus*, which

Sample and Station	Latitude; Longitude	Coll. Date	Relative abundances of *Calanus* species				PCR	
			C.fin N (%)	*C.hel* N (%)	*C.gla* N (%)	*C.hyp* N (%)	Failed	Total
Norwegian Sea								
Stn. 17 NW		Apr-97	6 (86)	-	-	1 (14)	0	7
Stn. 232 NW		Jun-97	30 (100)	-	-		0	30
Stn. 277 SW		Jun-97	18 (100)	-	-		2	20
Stn. 409/410 Bodo		Jul-97	30 (100)	-	-		0	30
Stn. 440/441 SW		Jul-97	23 (100)	-	-		1	30
Stn. 49/50 SW		Apr-97	29 (100)	-	-		1	30
Stn. 44			29 (100)	-	-		1	30
Stn. 8			29 (100)	-	-		1	30
Lurefjorden	60° 41.0N; 5° 10.5E	Oct-95	4 (36)	3 (27)	2 (18)	2 (18)	4	15
Masfjorden	60° 52.3N; 5° 24.7E	Oct-95	11 (73)	4 (27)	-	-	0	15
Oslofjorden	59° 48.0N; 10° 34.0E	Nov-95	12 (92)	1 (8)	-	-	2	15
Gulf of Maine	43° 49.8N; 67° 88.2W	Oct-97	12 (80)	3 (20)	-	-	0	15
Arctic	78°N; 160°W	Aug-98	-	-	-	9 (100)	6	15

Table 5-3: Relative abundances of *Calanus* species in zooplankton samples collected from several N. Atlantic and one Arctic region, based on species' identification using the multiplexed SS-PCR protocol. Individuals from the Norwegian Sea were copepodite stages CI, CII, or CIII; other samples used females or copepodite stage CV. Percentage values of relative abundance are given in parentheses.

could not be unambiguously assigned to either sibling species group based on this analysis (Figure 5-2).

DISCUSSION

MtDNA sequence variation, and mtCOI in particular, has been shown to be useful in resolving evolutionary relationships among closely-related species groups for a wide range of taxa (Brown et al. 1994; Lunt et al. 1996; Harasewych et al. 1997; see Avise 1994). For calanoid copepods, mtCOI sequence variation clearly resolves evolutionary relationships among the most closely related species (Bucklin et al. 1999, unpubl.).

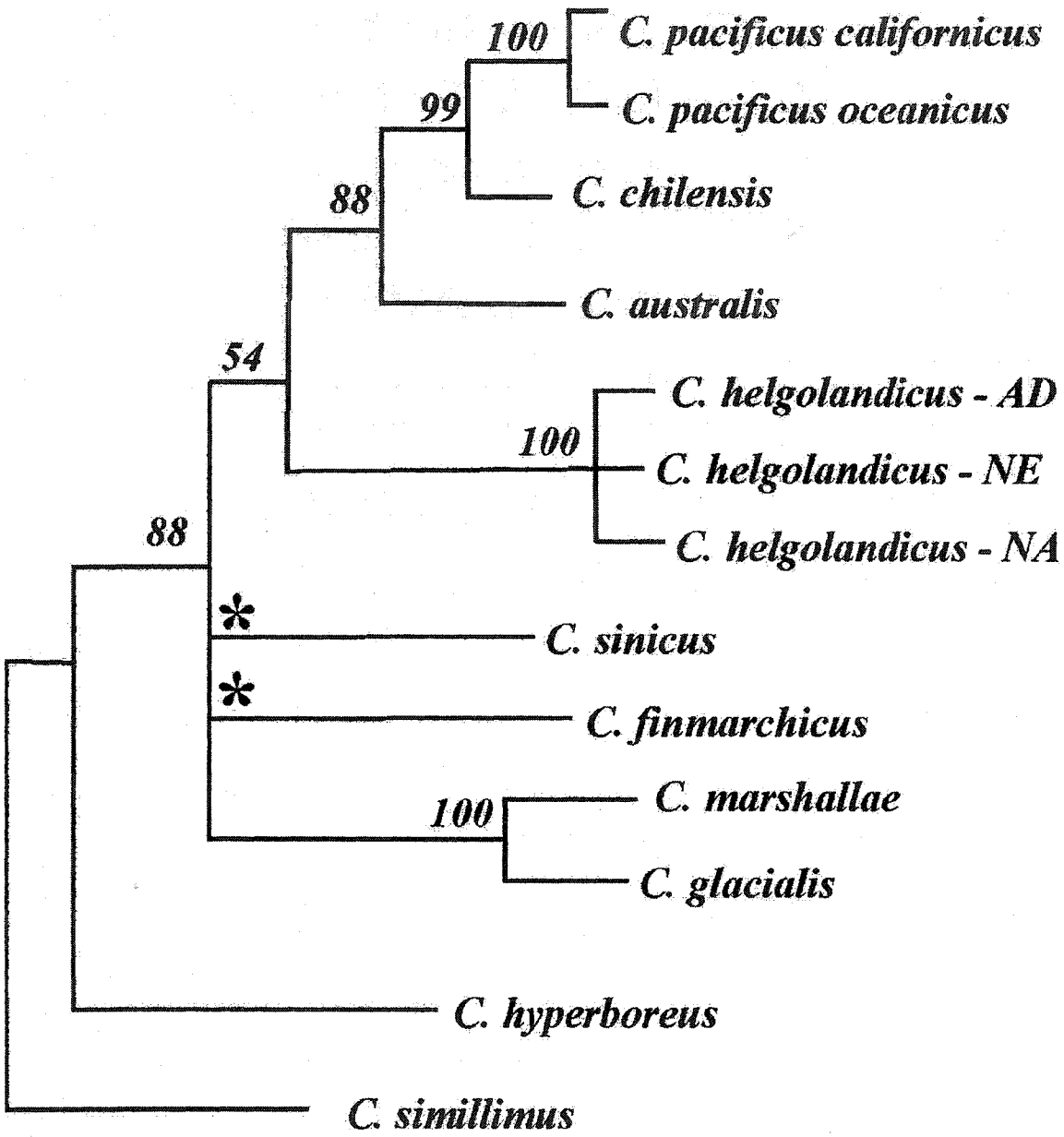

Figure 5-2: Phylogenetic reconstruction of evolutionary relationships among sibling species based on the mtCOI nucleotide sequences, using all bases. Samples of *C. helgolandicus* were collected from three regions: Adriatic Sea (AD), the Northeast Atlantic (NE), and the central N. Atlantic (NA); see Table 5-1 for collection information. Tree reconstruction was by neighbor joining (Saitou and Nei, 1987) using Tamura-Nei distances (Tamura and Nei, 1993) and alpha = 2.0; the tree was bootstrapped 1000X. Branch-points with bootstrap values < 50% were collapsed (shown by asterisks). Numbers in italics at branch-points are bootstrap values.

Importantly, the gene appears to evolve rapidly enough to distinguish even the most closely-related sibling species, suggesting that it will be useful to reveal cryptic species with this group.

The use of molecular data for identification of sibling species implies an evolutionary species' concept (i.e., species can be discriminated based on the level of genetic divergence between them). However, it should be noted that species cannot be delimited or defined solely on the basis of mtDNA sequence variation. There is no a priori relationship between genetic divergence and speciation, especially for mitochondrial traits which (by virtue of their clonal matrilineal transmission) are not directly linked to reproductive isolation and speciation events (see Avise 1994). Phylogenetic examination of the evolution of the mtDNA sequence within the target taxon is desirable. Following confirmation that mtDNA sequence variation is a diagnostic, stable, and accurate indicator of species identity, the molecular data are useful for taxonomic identification and uniform standards of species' identification - along with morphological, morphometric, and ecological characters.

mtCOI sequence differences among 19 calanoid copepod species of six genera ranged from 8 - 21% for the selected mtCOI gene portion (Bucklin et al. 1999, unpubl.). MtCOI sequence variation clearly discriminated even the most closely-related species and resolved evolutionary relationships among species within a genus and among some genera. Several previous population genetic studies of calanoid copepods have found mtCOI sequence variation within species of 1 to 2% (Bucklin et al. 1998a, 1999, 2000a, unpubl.). Our use of consensus sequences based on analysis of pooled individuals does

not allow us to evaluate levels of intraspecific variation for most of the *Calanus* species in the present study. The exceptions are *C. pacificus*, for which we examined two subspecies, and *C. helgolandicus*, for which we examined three populations considered by Fleminger and Hulsemann (1987) to represent possible subspecies. The analysis of *C. helgolandicus* was intended only to ensure that the SS-PCR primer site was conserved among different populations. Further analysis will be required to determine whether the species exhibits significant population genetic structure.

Despite some sequence variation within each species, mtCOI is a useful gene for molecular systematic identification and discrimination of calanoid copepod species that lack diagnostic morphological characters. In particular, mtCOI sequence variation is suitable for the design and optimization of an SS-PCR protocol, which requires identification of unique and diagnostic sequence regions for each species (see Bucklin et al. 1997, 1998a, 2000a). For SS-PCR to be accurate and easy to use, the sequences must differ sufficiently among species to allow identification of regions suitable for primer design (see Rychlik 1992). For SS-PCR to be multiplexed, the primers must be located at different positions along the sequence to produce amplified products of different sizes, which can be discriminated by agarose gel electrophoresis (Gibbs et al. 1989). The sequenced mtCOI gene portion included several short regions that differed between *Calanus* species by ~30% of the nucleotides, and that exhibited little or no variation within species.

The primary advantages of the two-step SS-PCR are low cost and increased efficiency. This is particularly desirable, since hundreds to thousands of individuals may need to be

identified to document species distribution and abundance at high spatial and temporal resolution - especially for zooplankton species (such as *Calanus* species) that are numerically predominant and geographically widespread. At minimum, a randomly-selected subset of samples should be molecularly assayed to confirm species' identity and distribution.

The multiplexed, competitive SS-PCR protocol is rapid, simple, and accurate for discrimination of the four *Calanus* species in the N. Atlantic. It was tested on copepodites and adults from regions spanning the geographic range of each species and yielded unambiguous species' identifications for *Calanus* species individuals from the Norwegian Sea, Norwegian fjords, Gulf of Maine, and Arctic Ocean. Frequencies of failed PCRs were high (i.e., >10%) in several cases, perhaps due to poor sample preservation. Importantly, the protocol never yielded more than one SS-PCR product. However, *C. helgolandicus* from Norwegian fjords gave a second, artefactual band that differed from any species-specific product band. This underlines the necessity for each set of PCR reactions to include both positive controls for each species and a negative control.

The protocol is designed for use by biological oceanographers in routine species' identification of individuals. It can also be applied to DNA purified from pooled, unsorted (alcohol preserved) samples, to determine which *Calanus* species may occur in a region. Alternatively, the protocol may be applied to a subset of individuals or samples, to spot-check for species' composition and identification errors. SS-PCR should be usable by researchers and technicians not widely trained in molecular techniques and

requires little equipment and specialized laboratory apparatus. SS-PCR may be implemented with any PCR machine, agarose gel electrophoresis and power supply, and UV light source (see Appendix).

Lindeque et al. (1999) published a molecularly-based protocol for identification of *C. finmarchicus, C. glacialis*, *C. hyperboreus*, and *C. helgolandicus*, suitable for all life stages, based on a three-step protocol of PCR amplification, restriction enzyme digestion, and agarose gel electrophoresis. Fortuitously, both the Lindeque et al. (1999) protocol and this SS-PCR protocol have been used to identify *Calanus* species collected from Lurefjorden, Norway. Although the collections were made in different years, the findings were similar - indicating that both protocols provide accurate identification of individual *Calanus* species. There may be some advantage to the two-step SS-PCR protocol, which has higher through-put of samples with fewer steps and avoids the expense and biochemical fragility of restriction enzymes. However, both protocols are appropriate for use by oceanographers and ecologists without molecular training.

Bucklin et al. (1999) published a multiplexed, competitive SS-PCR protocol for identification of *C. finmarchicus, C. glacialis*, and *C. helgolandicus*. The protocol had one important error: the sequence and species-specific primer identified as *C. glacialis* have now been confirmed to be *C. hyperboreus*. The protocol described in this study corrects this error, and includes confirmed individuals of both *C. glacialis* and *C. hyperboreus*. In addition, the *C. finmarchicus* species-specific primer is now based on the coding mtCOI sequence, rather than a putative pseudogene (see Bucklin et al. 1999).

A previous study by Bucklin et al. (2000a) used mt16S rRNA sequence variation to examine the distribution and abundance of *Calanus* species in three fjords of western Norway (Lurefjorden, Masfjorden, and Sognefjorden) and one fjord in eastern Norway (Oslofjorden). Somewhat surprisingly, *C. finmarchicus*, *C. helgolandicus*, and *C. glacialis* were found to co-occur in two fjords (Lurefjorden and Masfjorden), and both *C. finmarchicus* and *C. helgolandicus* occurred in two fjords (Sognefjorden and Oslofjorden). Unexpectedly, *C. glacialis* predominated in Lurefjorden; the persistence of *C. glacialis* in fjord environments was previously unsuspected (Stein Kaartvedt, University of Oslo, pers. comm.). The SS-PCR protocol was tested on the same samples from three of the fjords, yielding similar findings for relative species abundances. The similarity in results between species identification based on DNA sequencing (Bucklin et al. 2000a) and that based on SS-PCR helped confirm the accuracy and reliability of the rapid protocol.

SS-PCR has been used to discriminate two sibling species, *Pseudocalanus moultoni* and *P. newmani* (Copepoda: Calanoida), which co-occur over large portions of their geographic ranges (Frost 1989). The two species are readily distinguished by mtCOI sequence variation (Bucklin et al. 1998a, 1999, 2000b). Similarly to this study, species-specific oligonucleotide primers were designed from the mtCOI sequences and optimized for use in a competitive, multiplexed SS-PCR (Bucklin et al. 1999). SS-PCR was used to assay thousands of individual copepods in order to determine the seasonal evolution of the species' abundances over Georges Bank (Bucklin et al. 2000b). Patterns of mtCOI sequence variation for *Pseudocalanus* species were similar to those of *Calanus*: sequences of conspecific individuals differed by about 2%; *P. newmani* and *P. moultoni*

differed by about 30% (Bucklin et al. 1997). MtCOI may be a useful gene for identification and discrimination of calanoid copepods generally; preliminary studies suggest that mtCOI may contain unique oligonucleotide regions that may be used to design SS-PCR protocols for all ~2,400 species of calanoid copepods (Bucklin et al. 2000b; Bucklin, unpubl.).

The mtCOI gene tree for ten species of *Calanus* resembles in most respects both the morphologically-based view of the evolutionary relationships within the genus (Frost 1974; Fleminger and Hulsemann 1977, 1987) and previous molecular phylogenetic analysis based on mt16S rRNA (Bucklin et al. 1992, 1995). This concordance, especially with the 16S rRNA tree, increased our confidence in the use of mtCOI as a molecular systematic character for the genus. The mtCOI gene tree resolved the two sibling species groups, except that the relationship of *C. sinicus* and *C. finmarchicus* were not statistically resolved (i.e., bootstrap values were < 50%). The lack of resolution of species in the *helgolandicus* group was also observed for the mt16S rRNA tree (Bucklin et al. 1995). It is possible that these species are more distantly-related, making resolution of their relationships more difficult. It is also possible that both mt16S rRNA and mtCOI are too variable to resolve this group; a more slowly-evolving gene may be needed to reconstruct the evolutionary history of the genus, especially of the *helgolandicus* group.

In part because of relatively rapid evolution of the gene, mtCOI sequence variation may help resolve questions of the taxonomic significance of geographic variation for some *Calanus* species. For example, mtCOI clearly resolved genetic differences between geographic populations of *C. pacificus*, which are considered to represent distinct sub-

species (Jaschnov 1955; Bradford 1988). Individuals of *C. pacificus californicus* and *C. p. oceanicus* differed in mtCOI sequence by nearly 3%. In addition, it seems possible that mtCOI can resolve population genetic structure: samples of *C. helgolandicus* from the eastern and central N. Atlantic and the Adriatic Sea differed by 0.5 to 0.8% of the mtCOI sequence. Populations of *C. helgolandicus* were examined in light of Fleminger and Hulsemann's (1987) conclusion that there was significant morphological differentiation across the species' range. Although the differences in mtCOI sequences between geographic populations of *C. hegolandicus* are small, they may be useful for examining the genetic consequences and systematic significance of geographic variation in this species.

Molecular analysis of marine zooplankton is likely to continue to reveal taxonomically-significant genetic partitioning within species, including the discovery of cryptic species (e.g., Bucklin et al. 1996). This may be especially true for calanoid copepod taxonomy, which is plagued by numerous sibling species assemblages that are discriminated by subtle morphological characters. Molecular assessment has revealed significant genetic divergence among sibling species of calanoid copepods. Sequence variation of mtCOI regions is particularly useful to: 1) reconstruct the evolutionary and phylogenetic history of a speciose genus, 2) infer the boundaries of species and genera, and 3) identify and discriminate species with few or subtle morphological characters. In particular, competitive SS-PCR is useful for identifying the species of an individual copepod of any size or life stage. SS-PCR may also be useful for the analysis of DNA purified from unsorted zooplankton samples to determine the presence or absence of particular species in the assemblage.

A caution for biological oceanographers is that our ability to understand the community dynamics of planktonic assemblages may depend on our ability to accurately measure species' diversity and discriminate individuals of morphologically- and systematically-similar species. The myth of "functional groups" in marine zooplankton may mask a complex set of interactions among species that effectively partition the ocean along temporal and spatial boundaries that are unseen by typical analyses. Molecular systematic assessment using simple protocols may be useful and necessary to understand the population dynamics of target species and the community dynamics of planktonic assemblages.

NOTE

This study was published as: Hill RS, Allen LD, and Bucklin A (2001) Multiplexed species-specific PCR protocol to discriminate four N. Atlantic *Calanus* species, with a mtCOI gene tree for ten *Calanus* species. Marine Biology 139: 279-287.

CHAPTER VI

CONCLUSIONS

This study presents evidence that geographic populations of *Acartia tonsa* do not constitute a single species. Even some adjacent populations (i.e. Great Bay, NH to Buzzards Bay, MA) are genetically distinct and failed to interbreed in laboratory culture. In addition to genetic differences, these two populations appear to show distinct physiological responses to temperature in terms of survival, egg laying and hatching rates, and production of dormant eggs. For populations that did interbreed (Narragansett Bay, RI and Buzzards Bay, MA with Beaufort Inlet, NC), these was significant population genetic structure.

These finding raise interesting and pertinent questions regarding the evolution of estuarine copepods and copepods in general. Will these findings define a trend among calanoid copepods or obligate estuarine organisms, or is this a unique case? These findings also issue a word of caution to researches working with *A. tonsa*. Without a good understanding of the world wide population structure of *A. tonsa*, special effort should be made in identifying the source of individuals in future studies. Particular caution should be used when using *A. tonsa* as a model organism or bioindicator species (e.g., for environmental toxicity studies), since the high genetic diversity and potential for

rapid evolutionary adaptation may result in significantly different responses among different populations of *A. tonsa.*

Understanding the population structure and evolution of calanoid copepods is an important component to understanding the dynamic interactions and properties of marine ecosystems. However, the conserved morphology of the calanoid copepods has made it difficult to conduct such studies; even the identification of species is often unreliable and inconsistent. The application of molecular techniques allows rapid, reliable, and consistent identification of species. The use of these techniques also enhances the understanding of the diversity and structure of populations of calanoid copepods. The mtCOI analysis of 9 *Calanus spp.* have extended the results observed in *A. tonsa* populations to the open ocean, demonstrating that species in this very different environment may also show the same pattern of genetic divergence of morphologically indistinguishable species.

The use of molecular techniques to study the evolution and diversity of calanoid copepods has been limited and many questions remain unanswered. Has the diversity of calanoid copepods been grossly underestimated? Are most currently identified species really groups of cryptic species? The truth is probably that some groups are more diverse than previously thought while others are not. It is highly likely however, that the current estimates of diversity in calanoid copepods, based mainly on morphology, are low and that new species and complex population structures will continue to be unveiled. Understanding the environmental dynamics and species interactions that control the

levels of diversity and influence population structure and speciation in calanoid copepods will be a key part of understanding marine ecosystems. It is already becoming evident that there is significant difference in the population structures of calanoid copepods in the open ocean, in estuaries, and in freshwater lakes. The application and continued development of molecular applications will enhance, rather than replace, current research in the field.

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