GENETIC, MORPHOLOGICAL AND ECOLOGICAL RELATIONSHIPS AMONG POPULATIONS OF THE CLAM SHRIMP, *Caenestheriella gynecia*

by

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Abstract

GENETIC, MORPHOLOGICAL AND ECOLOGICAL RELATIONSHIPS AMONG POPULATIONS OF THE CLAM SHRIMP, *CANESTHERIELLA GYNECIA*

by
Jonelle I. Orridge

Advisor: Professor John R. Waldman

Little is known about the ecology of the clam shrimp, *Caenestheriella gynecia.* *Caenestheriella gynecia* was first discovered in 1939 in a single pool in Oxford, Ohio. Schmidt and Kiviat (2007) reported four new localities of *C. gynecia* in New York and New Jersey, three within the Hudson Valley of New York and one in northeastern New Jersey. *Caenestheriella gynecia* may have originated from a very small founder population due possibly to unusual dispersal vectors from its natural range to the west, in Ohio. Egg samples and hatched individuals were obtained from all study sites and specimens were raised in the lab to estimate several growth and survivorship traits. In the field, puddle habitats were observed between the months of May and August where water quality parameters (i.e., dissolved oxygen, temperature, conductivity and pH, and nutrient composition) were recorded. Genetic comparisons across the study sites were made using nuclear DNA sequencing and random amplified polymorphic DNA (RAPD) analysis. The results of this study presented a wide range in the hydro-chemical and physical characteristics of the ephemeral pools in which *C. gynecia* seem to tolerate.

Morphologically, New Jersey and Massachusetts populations possessed meristics counts within the range of those discovered by Mattox in 1950. However, I recommend the placement of the New York population within the *Cyzicus* genus as their meristic

measurements fell outside the range for *Caenestheriella*. RAPD results revealed the presence of more than one clone in puddles containing *C. gynecia* although mtDNA sequencing did not reveal any genetic variation within or among populations. The lack of males within *C. gynecia's* population and low levels of genetic variability support the clonal nature of a strictly parthenogenetic species. These investigations provide a substantial extension of fundamental knowledge of this poorly understood species.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my mother, Lorna Orridge. She was my first field assistant and without her help and guidance throughout this journey, I would not have made it to the end.

I would like to thank my advisor, Dr. John R. Waldman and my committee members, Drs. Stéphane Boissinot, Robert Schmidt and Pokay Ma for their questions, ideas and time. Special thanks go to my committee member, Dr. Frank Cantelmo whose enthusiasm in his aquatic courses sparked my interest to further my knowledge of aquatic ecosystems.

To my undergraduate assistants– Jessica Chandhok, Cynthia Komar, and Jacqueline Milander – for their help with field activities and laboratory assistance.

Lastly, but most recently, to my daughter for being my motivation during the last year. With this achievement, I hope to inspire you in all your future endeavors.

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INTRODUCTION AND BACKGROUND INFORMATION

Clam shrimp classification

Clam shrimp are an extant taxon of bivalved branchiopod crustacean bearing passing resemblance but no relation to bivalved mollusks. They are known from the fossil record, from at least the Devonian period and perhaps before (Webb 1979, Potts and Durning 1980). They were originally classified in the single order, Conchostraca, but have recently been broken down into the three orders Laevicaudata, Cyclestherida, and Spinicaudata (Figure 1) (Negrea et al. 1999).

Characteristics of the branchiopod class

Clam shrimp are small freshwater organisms belonging to the class Branchiopoda. An estimated 800 species in the class Branchiopoda have been found worldwide inhabiting freshwater ponds and lakes (Fautin and Follo 2005). This class consists of four living orders: The Anostraca ("fairy shrimp" or "sea monkeys"), Notostraca ("Tadpole shrimp"), Cladocera ("water fleas") and Conchostraca ("Clam Shrimp"). However, it is debatable as to whether cladocerans should be incorporated into the same class (Martin and Boyce 2004) (Figure 2). Their nervous, sensory and circulatory systems are simple – having phyllopodus or leaf like appendages that are used in gas exchange, locomotion and feeding. Most inhabit fresh or brackish temporary ponds and are absent from running water. Phyllopodus branchiopods typically have one (or two) generations each time habitat conditions are favorable.

Often only some eggs hatch at any given time while the remaining eggs are found in cyst banks.

Conchostracans *(Clam Shrimp)*

Morphology

Conchostraca are frequently referred to as clam shrimp because of their similarities to bivalved mollusks. All conchostracans have a bivalved carapace where a dorsal hinge joins to two valves and is controlled by a strong adductor muscle. Differences of conchostracans from anostracans and notostracans include the ability to be completely enclosed within the carapace and that they possess a reduced abdomen (Martin and Boyce 2004). According to the Museum of Paleontology at the University of California, Berkeley, conchostracans differ from the cladocerans in three primary ways: 1) Their appendages cover the entire thorax and abdomen region while the appendages of the cladocerans are found only on the anterior segments. 2) The body of conchostracans is entirely enclosed in the carapace whereas the head is excluded in the cladocerans. 3) The cladoceran's carapace is a single piece that wraps around to cover the animal, while the conchostracan's carapace is hinged, allowing opening and closing of the 'shell' (2005), (Figure 4).

General biology

Conchostracans occur on all continents except Antarctica. Even though there are specific habitats that differ by species, most might be encountered on the ground of ephemeral pools, i.e., vernal pools. It is not unusual to find them entirely dug into

mud like mussels (Eder 2005). Besides being filter feeders, these organisms can tear apart their food and will scavenge any organism in their environment (Martin and Boyce 2004). Clam shrimp are eaten by amphibians and other predators including notonectid hemipterans (backswimmers), mallards and other ducks; shore birds like the Killdeer, Great Blue Heron, Great Egret and other wading birds. The protein from clam shrimp provides important nutrition for migrating birds (Krapu and Reinecke 1992). They visit the vernal pools to quickly gather the nutrients they need to grow new feathers, migrate and lay their eggs. In Michoacan, Mexico, clam shrimp have been collected and used commercially as dry pet food (Martinez – Pantoja et al. 2002).

Life History

Clam shrimp are sexually dimorphic and are categorized as males and females on the basis of morphological dimorphism but now there are studies that show the "females" are actually functional hermaphrodites (Sassaman and Weeks 1993). The first two pairs of thoracic appendages in males undergo differentiation into claw-like claspers that they use to grab the shells of females and then hang on at right angles to the female's shell length (Martin and Boyce 2004). Zucker et al. (1997) provided anatomical evidence for androdioecy in the clam shrimp, *Eulimnadia texana*. They found the existence of both well-developed ovarian and testicular tissue in the gonads of the same individual, confirming the idea that parthenogenetically-reproducing females are actually hermaphrodites.

Conchostracans develop very quickly. Desiccation-resistant eggs are buried within the top several centimeters of soil. Two types of eggs can be produced, summer eggs and winter eggs. Summer eggs hatch almost immediately while winter eggs (also referred to as resting eggs) can endure periods of extreme heat, cold and desiccation. Resting eggs allow continuity in an environment that goes through periods of drying and freezing annually. Most hatch as a pear-shaped nauplius larva with three pairs of appendages and some species undergo remarkable changes in morphology in a single molt (Hann 1996).

Many factors influence the growth and reproduction of these animals, mainly fluctuations in their habitat. This includes the physical characteristics of the water body (i.e., temperature, pond size, inert suspended particles); chemical characteristics (salinity, pH, oxygen concentration, alkalinity, etc.); richness in food resources; number of times a pond/pool refills during different seasons; and interspecies interactions (competition, predation, dispersal agents). Another important factor is pond duration. Pond/pool duration can be influenced by average rainfall, evaporation rate (which is influenced by surface to volume ratio, daily temperature and relative humidity) and soil type. Clam shrimp that live in smaller ponds in areas with low average rainfall should experience a shorter total time available for development than those in larger ponds or in areas of higher rainfall. These shrimp should then have reduced longevity, faster growth, an earlier age at maturity and lower fecundity. Marcus and Weeks (1997) tried to show the effects of pond duration on the life

history traits of *Eulimnadia texana* but did not produce any results that correlate well with the evolutionary model, probably because of low sample size.

Distinguishing populations can be a difficult task because the species can go through cyclical successions of absence and presence in an area. They could be abundant for several consecutive years and then disappear for one or more years. It is not uncommon for clam shrimp to inhabit some puddles and be completely absent from those in close proximity. These stochastic distribution patterns may result from the mode of dispersal of their eggs. The resting eggs may be naturally transported by the wind, birds or insects that visit the pools to drink or breed and by human vectors, i.e., as all-terrain vehicles (ATVs) (NatureServe 2006).

OBJECTIVES

In this dissertation, I investigated and compared the ecological, morphological and genetic characteristics of a species of clam shrimp, *Caenestheriella gynecia,* from three localities: vernal pools in the Hackensack Meadowlands of New Jersey; vernal pools in Massachusetts; and vernal pools in Saugerties, NY. I also assessed the genetic structure among different populations and sub populations of the clam shrimp, *Caenestheriella gynecia*, generating information useful in assisting conservation biologists making management decisions concerning *C. gynecia* or other invertebrates inhabiting vernal pools in the New Jersey Meadowlands.

Chapter I provides an overview of the clam shrimp species, *Caenestheriella gynecia*. It addresses the general ecological, morphological and genetic characteristics of *C. gynecia* and describes the sites used in the study. In Chapter II, I report on several aspects of the life history characteristics of *C. gynecia*. In Chapter III, I compare the morphological relationships and life histories among populations of *C. gynecia*, within the previously mentioned states. Populations found in the Meadowlands are thought to be *Caenestheriella gynecia* on the basis of the absence of males and their ecology. If males were found, comparative shape of the female and male rostrum to the genera *Cyzicus* would determine true classification of the species. The purpose is to settle the argument that they are not in fact different species. Chapter IV addresses the specific objective of assessing the ecological aspects of vernal pools, found in three states – NJ, NY, MA – , which render them habitable or inhabitable to *C. gynecia.* In Chapter V, I seek to determine the genetic relationships between populations of *C. gynecia* found in vernal pools in the Meadowlands, Massachusetts and New York. As a parthenogenetic species, with no observed males, each locality should have little genetic variation when compared to each other and among pools found in the Meadowlands. Using the results from the genetic and morphological analyses of this study, Chapter VI will decipher the mating system of *C. gynecia*. The implications of this study are then summarized and provide general conclusions for the entire project.

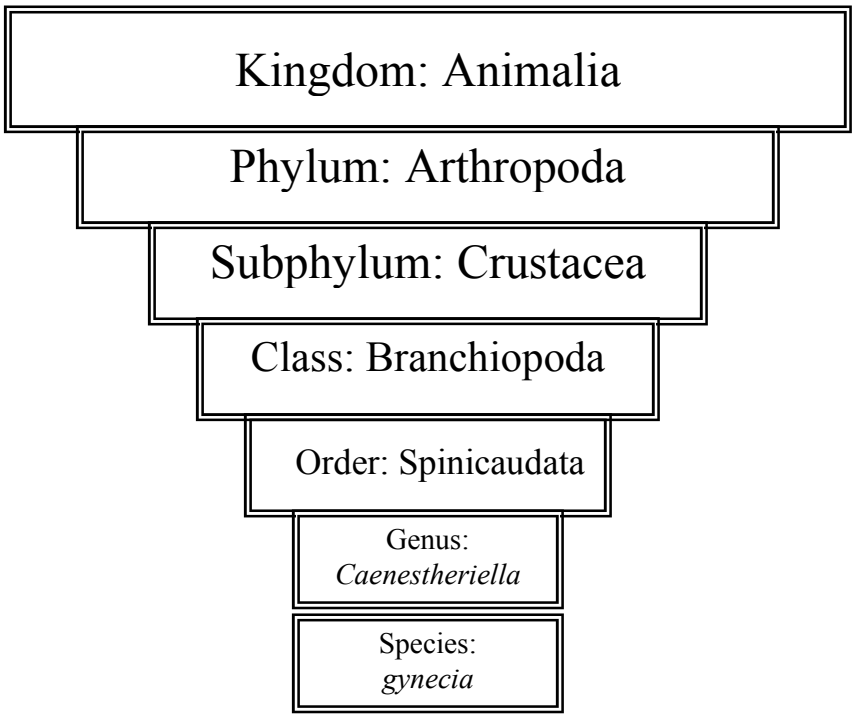


Figure 1. Scientific classification of the clam shrimp, *Caenestheriella gynecia.*

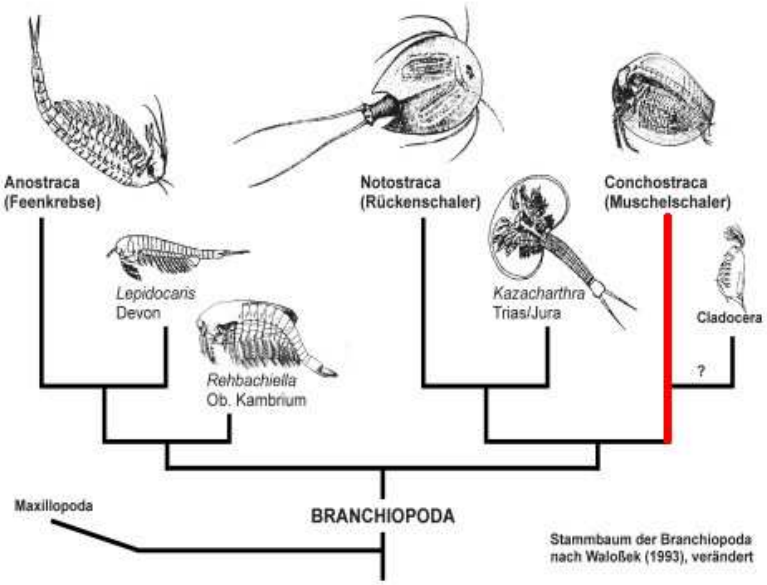


Figure 2. Tree showing the phylogenetic relationships in Branchiopoda.

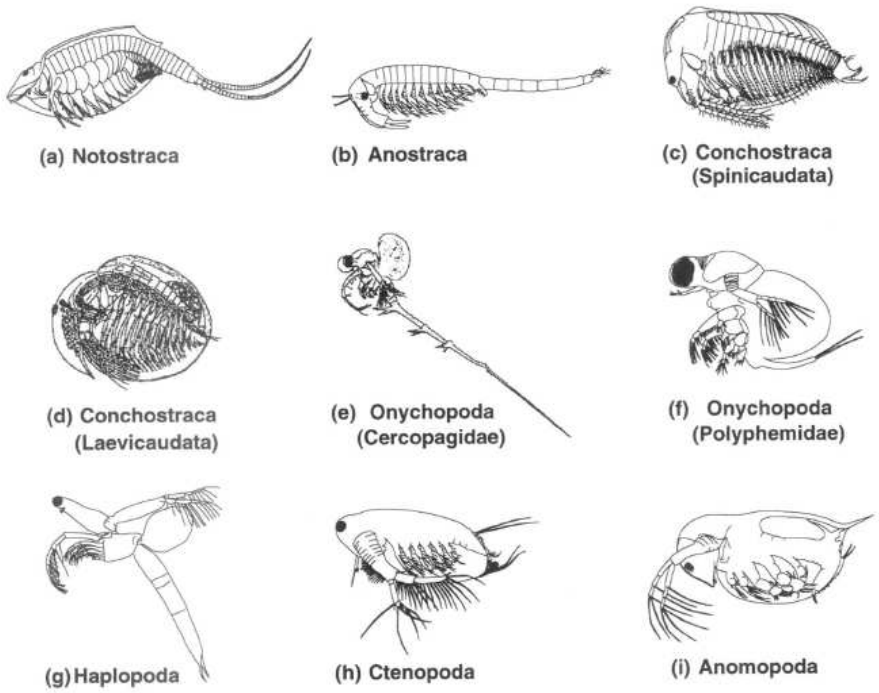


Figure 3. Branchiopod diversity (excerpted from Spears and Abele 2000).

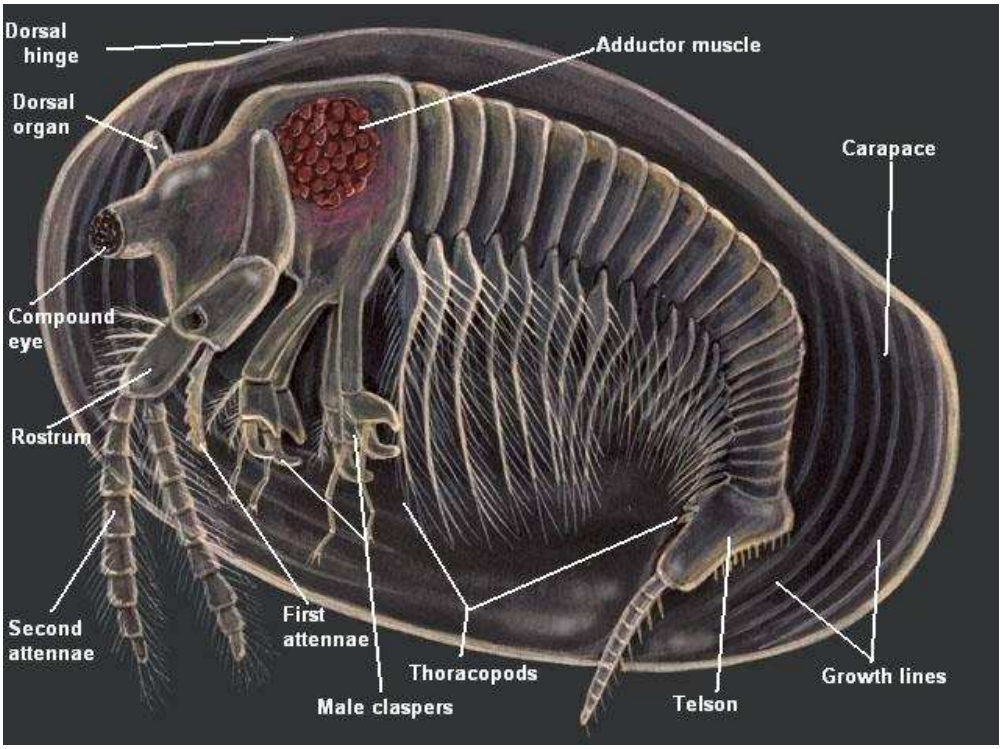


Figure 4. Conchostracan morphology (Meyers et al. 2006).

CHAPTER I: *CAENESTHERIELLA GYNECIA* POPULATIONS IN NORTH EAST AMERICA

INTRODUCTION

This project focused on *Caenestheriella gynecia* (suborder Spinicaudata; Figure 5). *Caenestheriella gynecia* Mattox 1950 is a poorly understood representative of the clam shrimp family Cyzicidae (Smith and Gola 2001). Cyzicidae is a small family of the clam shrimp Order Spinicaudata that is globally distributed (except for Antarctica). There are four genera, three in the American continent and one outside (*Caenestheria)*. The three North American genera are *Caenestheriella, Cyzicus* and *Eocyzicus. Cyzicus* is the largest genus with five species, one having the widest distribution of any North American cyzicid ranging from Mexico north to Canada and east to Pennsylvania and Maryland. The other four are found only west of the Mississippi River. *Eocyzicus* is limited to southwestern North America. *Caenestheriella* consists of three species: *Caenestheriella setosa, C. belfragei, C. gynecia*. *Caenestheriella setosa and C. belfragei* are found in Midwestern North America. *C. gynecia* is the only cyzicid besides *Cyzicus mexicanus* that are known to occur east of the Mississippi River, ranging from Ohio, Pennsylvania and New England (Smith and Gola 2001).

Little is known about the ecology of *C. gynecia. Caenestheriella gynecia* was first discovered in 1939 in a single pool in Oxford, Ohio. Schmidt and Kiviat (2007) reported four new localities of *C. gynecia* in New York and New Jersey, three within the Hudson Valley of New York and one in northeastern New Jersey.

The majority of clam shrimp species are sexually dimorphic. The first two pairs of thoracic appendages in males have differentiated into claw-like claspers which they use to grab the shells of females and then hang on at right angles to the female's long axis (Martin and Boyce 2004). There are studies that show that "females" are actually functional hermaphrodites possessing both well-developed ovarian and testicular tissues (Sassaman and Weeks 1993). Contrary to the norm for clam shrimp (sexual dimorphism), *Caenestheriella gynecia* is a parthenogenetic (asexual) species with no record of males. It is possible that males are rare and have yet to be discovered. Eggs are present in females between June and October and can remain viable without hatching for nearly 8 years. Adults can live up to 6 months (Mattox and Velardo 1950). Egg viability is affected by temperature and time required for development and hatching varies with changes in temperature as was shown by Mattox and Velardo (1950). Water persistence is necessary in a pool for a given amount of time in order for the animals to survive. Pond duration constrains the length of life and time available for the organism to reach reproductive age. Exact time of pond duration is varied and depends on the life history traits of the species (Marcus and Weeks 1997).

As a parthenogenetic species, the genetic variation of *C. gynecia* should be low. Fewer genetic alternatives result in asexual reproduction, whereas the DNA in offspring of sexual organisms undergo many gene conversions and crossing over, causing the individual chromosomes to become a mosaic of maternal and paternal materials (Charlesworth 2006). Another factor which would contribute to low genetic

variation is that the eastern populations of *C. gynecia* may have originated from a very small founder population due to a dispersal event from its natural range to the west (Schmidt and Kiviat 2007).

Caenestheriella gynecia is so little known that only five scientific papers have been published about it. It may have an ecological significance as a food source for migratory birds as some clam shrimp species are among the aquatic invertebrates that dominate the diets of female mallards in the Prairie Pothole Region (Krapu and Reinecke 1992). Its habitat is found in open, muddy puddles on manmade dirt roads. Although this environment is odd for a clam shrimp, it is a common habitat in the U.S. and it seems likely that it will continue to expand its range to other similar locations. Wherever it occurs, either as a native or non-native, it contributes a unique set of life history characteristics that are intriguing, but still poorly defined.

STUDY AREAS

Hackensack Meadowlands, NJ (MNJ)

New Jersey is divided into four to five different physiographic regions (depending on sources). Moving from north to south there is the Valley and Ridge, the Highlands, the Piedmont, and the Coastal Plain (further separated into the Inner Coastal Plain and Outer Coastal Plain). The Hackensack Meadowlands are located in Bergen County, NJ, which is found in the Piedmont region. The Piedmont province is approximately 1,600 square miles and makes up about one-fifth of the state. This region is highly urbanized and was home to the former glacial lake, Lake Passaic. In its place is the

Great Swamp which harbors a variety of vernal pool complexes (Lathrop et al. 2005; New Jersey Geological Survey).

The Hackensack Meadowlands are composed of approximately 8,300 ha of wetlands, uplands and developed areas in northeastern New Jersey (Kiviat and MacDonald 2004). It is approximately 11 km west of the borough of Manhattan, New York and 8 km north of Newark, New Jersey. The Meadowlands are in the lower Hackensack River drainage, which flows into the northern end of Newark Bay. Elevation ranges from 0 – 3 m above sea level in the wetlands to 30 – 50 m upland. Numerous habitats can be found within this large wetland complex. These include a variety of brackish tidal creeks, canals and ditches; tidal marshes; non-tidal marshes and hardwood swamps; and shrublands, woodlands and meadows found in areas that was once a solid waste landfill, low lying wetland fill or clayey or sandy soils. More urbanized habitats include industrial areas, residential yards, and parks (Kiviat and MacDonald 2004).

To fully understand urban biodiversity, it is important to study organisms of less studied habitats other than the traditional marshes which most people deem to be important. This is especially true for smaller invertebrates that inhabit small or temporary pools; storm-water ponds; and artificial habitats such as puddles created and maintained by consistent traffic of various road vehicles. The freshwater systems created as a result are composed of small habitable patches surrounded by large uninhabitable areas.

The study was conducted at a set of 10 rain puddles on the dirt surface of a gas pipeline road in the Empire Tract between Empire Boulevard and the Interstate 95 (I-95) (Figures 6-8). GPS coordinates of the puddles can be found in Table 1. The road at this locality is regularly used by ATV and other off-road vehicles, i.e., four wheelers, motorcycles, pickup trucks, sport utility vehicles (SUVs) and dirt bikes. It is hypothesized that the frequent activities of these vehicles created and maintain the puddle habitats (Schmidt and Kiviat 2007). The dirt road is lined by common reed, *Phragmites australis*, small trees and tall shrubs. It is elevated about 1.5 m above the surrounding marsh which was formerly tidal. I was informed by one of the SUV operators that during heavy rains, the road can be completely flooded with up to two feet of water. This illustrates a potential form of dispersal for the organisms. The dirt road is composed of clay-like material with a light reddish color which may have originated from red Triassic sandstone and shale found nearby (Schmidt and Kiviat 2007).

From what is known, in the Hackensack Meadowlands, *Caenestheriella gynecia* occurs only in the pools formed on the gas pipeline road in a two-thirds mile long section and is the only known locality for this species of animal in New Jersey (Schmidt and Kiviat 2007). It is possible that vernal pools formed in the Meadowlands support *C. gynecia* populations because of the reduced predation or competition that would have been present in larger permanent ponds.

Pittsfield and Lenox, MA (LPMA)

For genetic comparisons, specimens were taken (either directly or through collaborations) from several vernal pools located in Pittsfield and Lenox, MA (Figures 9 and 10). Puddles located in Lenox, MA occur at the edge of a large wetland in the Housatonic River floodplain along an abandoned dirt road. Soils within the puddles consist of a silt-loam mixture and ranges from slightly acidic to slightly alkaline (Smith and Gola 2001). The areas surrounding the puddles provide a dense canopy of pine, maple, elm and other trees, which shades them. Like the Meadowlands site, the dirt road is visited and the puddles are probably maintained by off-road vehicles. Puddles located in Pittsfield, MA contain soils that are a mixture of tunbridge (loamy, well-drained soils that formed in Wisconsin-age glacial till) and muck. Compared to the Lenox site, the pH of the water ranges from moderately to very slightly acidic. Direct sunlight is also reduced by a canopy of pin pine, hemlock, birch and oak trees (Smith and Gola 2001).

Saugerties, NY (SNY)

In July 2007, populations of *C. gynecia* were discovered by Erik Kiviat (Hudsonia Limited) in puddles on a dirt road in Bristol Beach State Park, Saugerties, New York, 73°55'58"W longitude, 42°06'33"N latitude (Figure 11). The park is one of the 10 sub-units of the northern Ulster Scenic Area of State Significance found in the Hudson River Valley. In 1997, 58 acres of Hudson River shorefront was added to the 53 acres of undeveloped park. The park consists of riverfront, meadows, woodland, marsh and tidal flat habitats. In addition, the park is interspersed with several ATV trails.

Sugar Loaf, NY (SLNY)

In September 2009, populations of *C. gynecia* were discovered at the Glenmere Reservoir and the Black Meadow creek. This site is located in Orange County, NY, in the towns of Warwick and Chester. Glenmere presents one of the largest contiguous wild areas in Orange County and consists of approximately 2 square miles of hardwood swamp, shale ridgelines, wide marsh, mossy bogs, vernal pools and open-water reservoir. Glenmere is found between the Wallkill and the Moodna watershed basins (Glenmere Conservation Coalition).

Figure 5. Study organism - *Caenestheriella gynecia.* Lateral view of living organism, head to left. Specimen collected from Meadowlands, NJ.

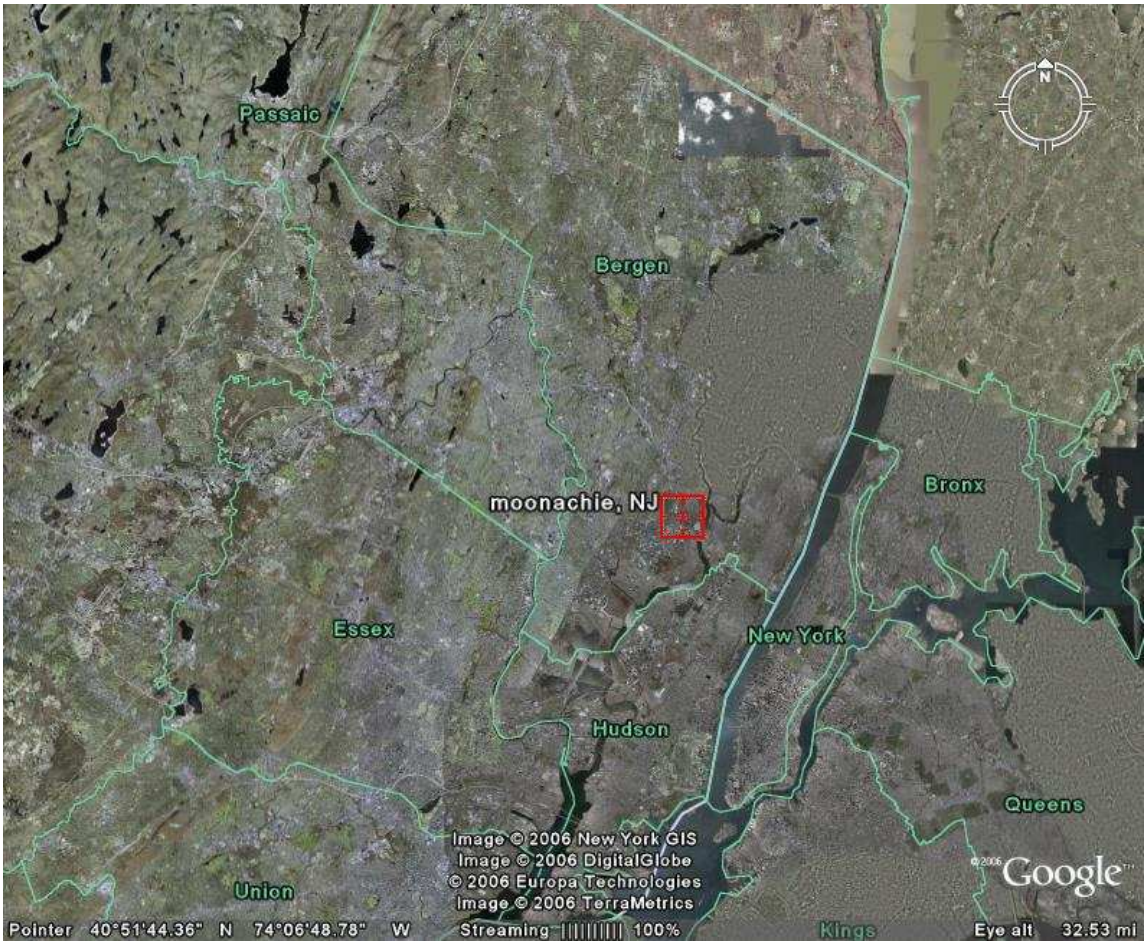


Figure 6. Aerial map of Bergen County indicating puddle site location. www.googlemaps.com

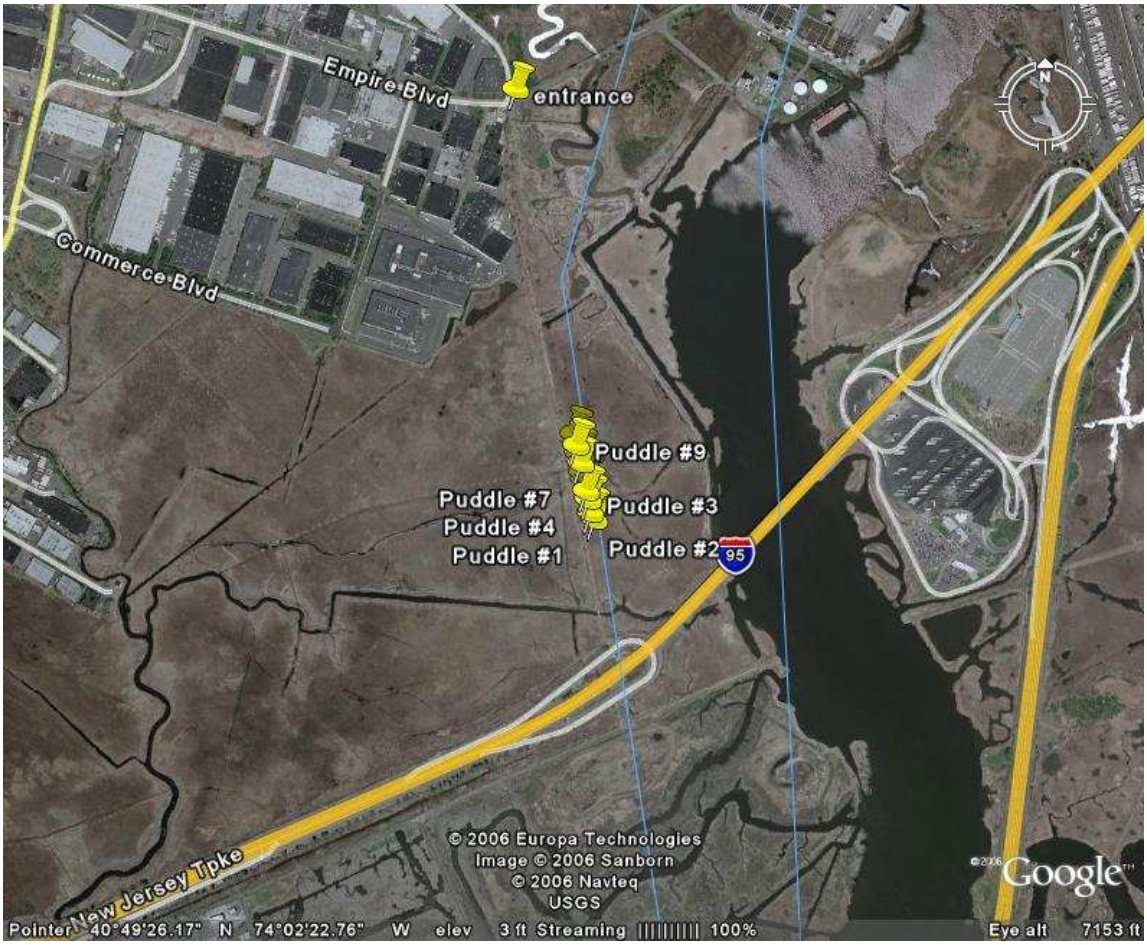


Figure 7. Aerial map showing sites located between Empire Blvd and I-95. www.googlemaps.com

Figure 8. Aerial map of puddle distribution on the gas pipeline road, Empire Tract, Bergen County, NJ. www.googlemaps.com

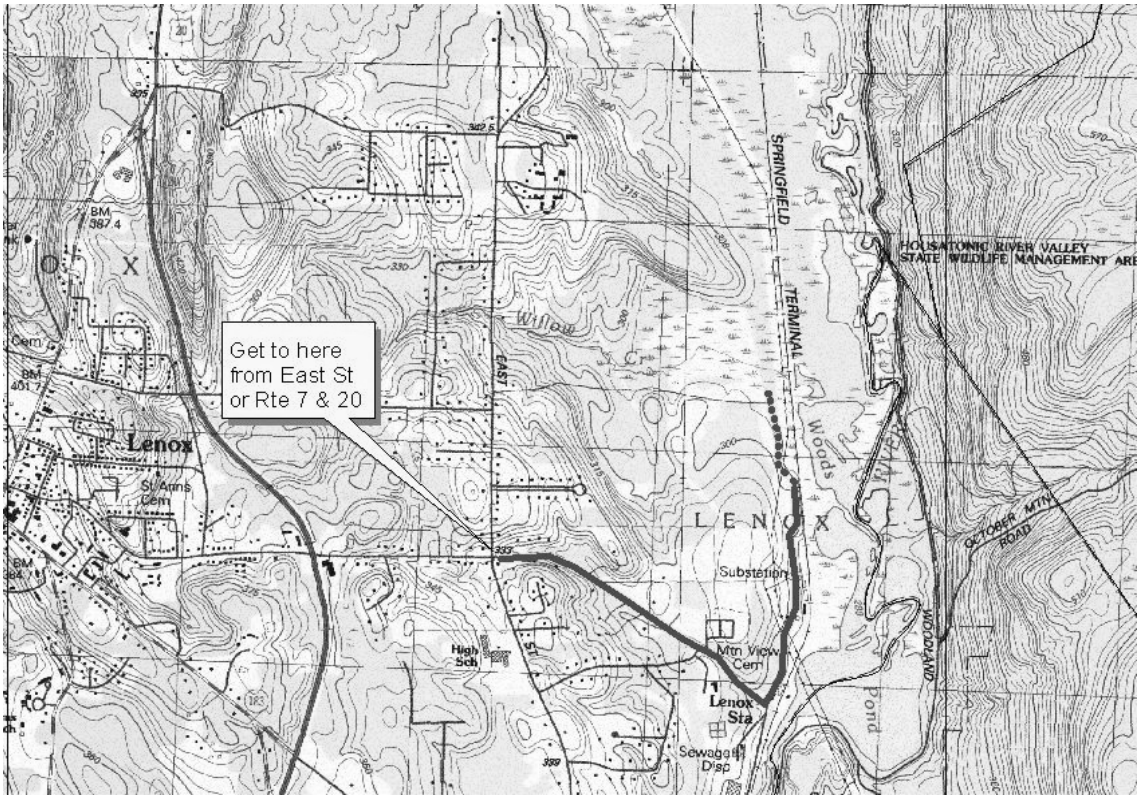


Figure 9. Topographic map showing Lenox, MA site. www.topozone.com

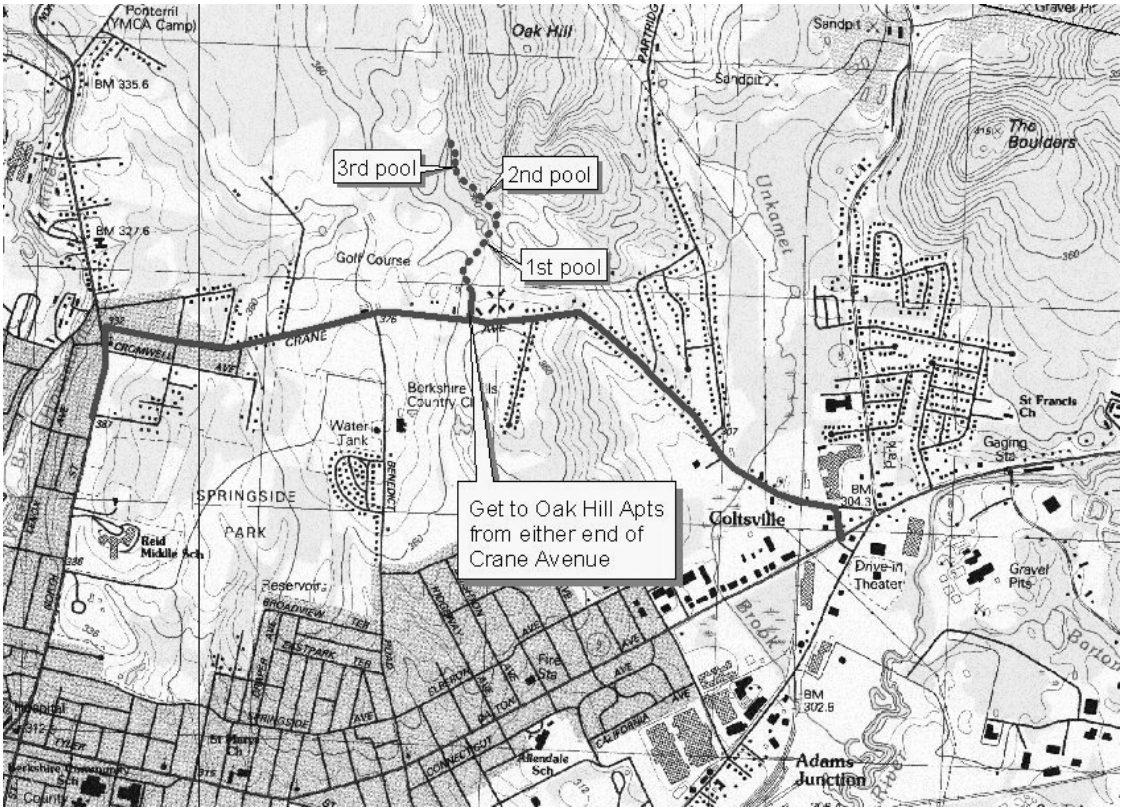


Figure 10. Topographic map of Pittsfield, MA site. www.topozone.com

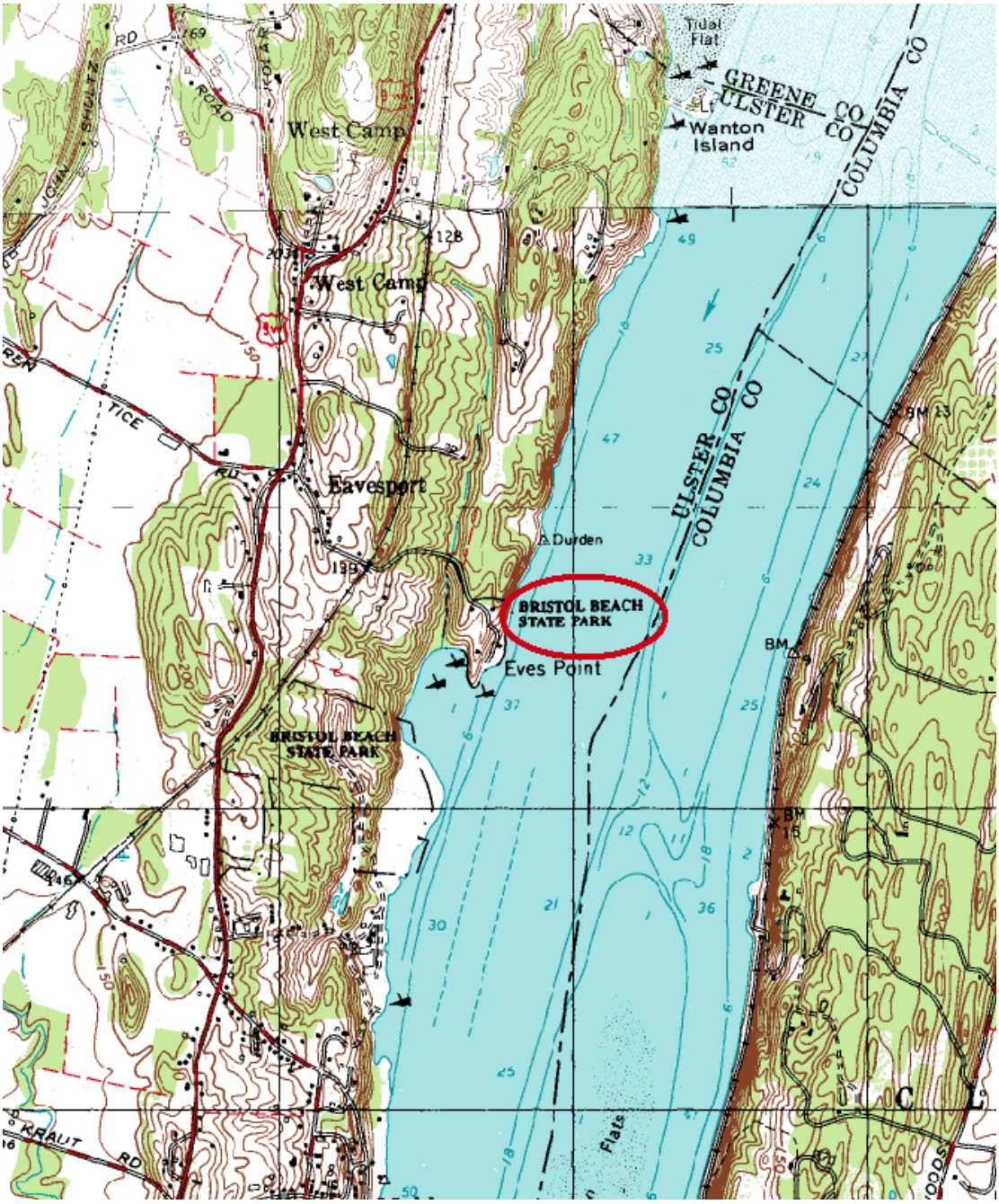


Figure 11. Topographic map showing location of Bristol Beach State Park, Saugerties, NY. www.topozone.com

Table 1. GPS coordinates of the 10 puddles monitored at the New Jersey site.

Puddle	Coordinates
1	N 40.82747
	W 74.04084
2	N 40.82715
	W 74.04072
3	N 40.82679
	W 74.04062
4	N 40.82656
	W 74.04049
5	N 40.82586
	W 74.04036
6	N 40.82526
	W 74.04020
7	N 40.82496
	W 74.04009
8	N 40.82473
	W 74.03997
9	N 40.82449
	W 74.03988
10	N 40.82422
	W 74.03985

Table 2. GPS coordinates of Massachusetts puddles used in study

Puddle	Coordinates
Lenox, MA	N 42.21731 W 73.14731
Pittsfield, MA	N 42.28637 W 73.13438
	N 42.28970 W 73.13563
	N 42.29046 W 73.13578

CHAPTER II: LIFE HISTORY CHARACTERISTICS AMONG POPULATIONS OF *CAENESTHERIELLA GYNECIA* IN MASSACHUSETTS, NEW JERSEY AND NEW YORK

INTRODUCTION

Life history of the Crustacean Order

Crustacean larvae can be one of three types – zoeae, megalopae, and nauplii – characterized by the appendages they use for swimming (Williams 1994). Zoeae and megalopae are only found in members of Malacostraca, which includes decapods (crabs, lobsters and shrimp), stomatopods (mantis shrimp) and euphausiids (krill). The carapace of a zoea covers the head and the forward portion of the thorax. Zoeae have compound eyes, thoracic segments with the appendages belonging to the segments, as well as some abdominal segments. Zoeae use their thoracic appendages to swim. Megalopae are sometimes called post larvae and are intermediate between the planktonic and benthic periods of their life cycle. Megalopae swim using their abdominal appendages or pleopods (Villamar and Brusca 1988).

There are a number of crustacean classes that share the nauplius larval form during their early hatching stages. These include the euphausiid and peneid shrimp, barnacles, copepods and branchiopods (Passano 1961, Williams 1994). Nauplii are morphologically characterized by the presence of three sets of appendages belonging to the cephalic segment – first antennae (antennules), second antennae, and mandibles. They swim using the antennules and antennae. Nauplii have a cephalic shield or the beginnings of the dorsal carapace, and no segmentation on the trunk. As the nauplius feeds and grows, it gradually changes into the adult form – the body

becomes segmented, or jointed, and additional limbs develop. They also possess a single eye in the anterior portion of their body which is retained in adults (Schram 1986, Waggoner 2010).

Life History of Caenestheriella gynecia

Individual variation in life history traits influences an organism's survival and reproduction while the actions of a set of individuals determine the dynamics of the population (Brommer 2000). During an individual's lifespan, it must undergo a series of transitions beginning with hatching, experiencing a form of metamorphosis, and reaching maturity. All of these stages are usually determined by a minimum age and size of an individual and is known as the developmental threshold. Trade-offs can occur between these two traits. An individual could make the transition to maturity earlier in age and at a smaller size, thereby increasing its chances of survival to reproductive age. However, the individual would have low fecundity due to its small size. On the opposite end, an individual could delay this transition and reach reproductive age at a larger size but this increases the risk of not being able to reach maturity due to other environmental factors (i.e., temperature, time of season, risk of predation) (Day and Rowe 2002).

Population persistence relies on how individuals allocate their energy and resources to reproduction and how the different age classes (larvae, juvenile and adults) respond to their environment (Brommer 2000). For branchiopods, the duration of ponded water in its vernal pool habitat affects their life history. A fast drying pond should

select for individuals that would be small in size and produce a lot of eggs early, while longer-lived ponds would select for larger individuals that have higher total lifetime fecundity (Marcus and Weeks 1997). It is therefore necessary to understand the life history traits of *C. gynecia* so that this information can be used in managing its habitat.

Of the literature available on *C. gynecia*, only two studies provide detail on some of its life history traits (Mattox 1950, Mattox and Velardo 1950) while the others just touch on it (Emberton 1980, Smith and Gola 2001). Mattox (1950) describes the hatching of *C. gynecia* eggs as "the continuous imbibing of water until the outer shell is broken where the nauplius is therefore released from the inner membrane." Growth of the nauplii is rapid and can increase in size from 0.37 mm (at time of hatching) to 0.48 mm in three hours, with a shell appearing after 96 hours. The nauplii also undergo several morphological changes with appendages starting to appear after 10 hours (Figure 12).

Mattox and Velardo's (1950) work on *C. gynecia* focused on the conditions necessary for egg hatching to occur. They found that the main determinant factors were temperature (between 13° and 38° C) and duration of desiccation where drying tends to retard the development and hatching of the eggs. In addition, an individual completed its life history, from egg to adult in 23 days. In this chapter, I report additional findings on these and new aspects the biology of *C. gynecia*: growth rates, daily egg production, daily molts, growth ring production and the interactions among these variables.

METHODS

Collection

Samples were obtained from dormant cysts in soil collected from the MNJ, LPMA and SNY study sites. The soil was stored in air-tight plastic bags until needed for hydration. Immature individuals isolated from field collections were monitored in the lab as well. Clam shrimp were considered immature if no eggs or claspers were visible.

Aquaria

Clam shrimp were reared and larvae were collected in the laboratory by hydrating 300 mL of dry soil with 17 L of aged tap water, added to a 38 L aquaria with light aeration. Aquaria were maintained at a constant temperature of 29 ± 2°C and kept under constant lighting using sunlight-simulating fluorescent bulbs. The only food source provided was a mixture of crushed algal pellets (Wardley Spirulina discs) and fish food (Tetramin goldfish flakes). Additional food may have resulted from the naturally occurring algae and bacteria that colonize the tanks.

For determination of individual growth and fecundity, larvae were isolated in 120 ml glass jars filled with filtered water (to remove potential eggs) from their rearing tanks. They were isolated in these jars after 3 days (days since hatching) under constant lighting using sunlight-simulating fluorescent bulbs (Week et al. 1997). Aged tap water was added to the cups to replace any water lost by evaporation.

Life history

Hatching time was recorded from the date of hydration while age was recorded as the time since hatching. Hatched nauplii were removed from the aquaria to avoid multiple counts. Some nauplii were placed in 70% ethanol for genetic analysis. Some nauplii were placed in individual polystyrene jars for the observation of life history traits.

Daily growth measurements were performed in two ways, using an image analysis system (Leica Application Suite) and/or a Mitutoyo dial caliper. A specimen was removed from its jar and placed with a small amount of water into a petri dish. Two measurements were attained, a dorsal length (measured between the two ends of the elliptical shell) and a lateral length (measured from the umbone of the carapace to the opposite end of the shell) (Figure 13). The specimen was returned to its jar after measuring. Daily growth increments were then calculated for each shrimp by subtracting its size at day X from its size at day X + 1. Length measurements were measured starting on day 4 because it was established by Weeks et al. (1997) that daily handling of the shrimp increases mortality when the shrimp are small.

Eggs were counted using an image analysis system, Image J 1.38x (Weeks et al. 1997). Two lateral images were taken of each shrimp and the eggs were counted directly through the translucent carapace. In addition, any eggs found in the individual's jar after removal were counted. Age at maturity was determined by the

day at which developing eggs or eggs in the brood chamber were first noted in the "hermaphrodites."

Sex determination followed Weeks et al. (1997) as follows: males, if any, were identified by the formation of claspers on the first two pairs of phyllopods, the absence of claspers and the presence of eggs indicate females. Individuals that did not possess both claspers and eggs where characterized as females.

Longevity was determined by the length of time (in days) each individual survived and carapaces were preserved for ring counts. Average lifespan was determined. Molts were counted and collected/removed daily to determine the number of molts per day. Correlations between molts and egg production were examined. Ring counts were made on a subset of preserved carapaces of individuals used for dissection. Counts started from the point of origin (at the umbone) and ended perpendicularly on the ventral side of the clam shrimp. Using a dissecting microscope, and the Leica image analysis system when necessary, rings were counted consecutively starting at the umbone and ending at the ventral side of the carapace.

For individuals collected from the field, daily growth, number of rings, longevity, age at maturity and egg production measurements were made using the date of collection as day zero. All life history measurements were conducted in the laboratory.

Statistical Analyses

Analysis of Variance (ANOVA) was performed to detect if there is a significant difference between the different parameters mentioned above (number of molts, longevity, age at maturity, etc.) among populations using JMP v. 8.

RESULTS

Hatching time

Under these laboratory conditions, *C. gynecia* eggs began hatching 5 days after hydration, with a hatching period lasting from 4 to 33 days after hydration. The number of larvae found started to decrease 5.5 days after hydration. The three populations were found to hatch differently by two criteria: the day at which 50% of larva hatched and the day at which 100% of the larva hatched. Fifty percent of the New York (SNY) larva hatched 4.8 days after hydration with all hatching 7.1 days after hydration. Fifty percent of the Massachusetts (LPMA) larva hatched 5.3 days after hydration with all hatching 12.6 days after hydration. Fifty percent of the New Jersey (MNJ) larva hatched 6.9 days after hydration with all hatching 33 days after hydration (Figure 14).

Daily growth and ring count

Larvae

Daily dorsal growth ranged from -0.0030 – 0.533 mm/day with an average growth of 0.145 mm/day and a maximum dorsal carapace size reaching 5.95 mm. Daily lateral growth ranged from -0.0130 – 0.405 mm/day with an average growth of 0.097

mm/day. Negative values indicate a reduction in size. Larvae produced, on average, 0.210 rings/day with one individual producing 16 rings ($\overline{x}$ = 4.8) during its lifespan (Table 3). There was a significant positive relationship between the dorsal (r^2 = 0.627, n = 27, P <0.0001) and lateral (r^2 = 0.422, n = 21, P = 0.0014) measurements and the number of rings on the carapace of the clam shrimp, with the number of rings increasing with an increase in dorsal and lateral growth (Figure 15).

MNJ population

Individuals collected from the field grew on average 0.052 ± 0.13 mm/day dorsally and 0.041 ± 0.13 mm/day laterally with a maximum carapace size of 9.12 mm. The clam shrimp produced 0.40 ± 0.44 rings per day with an individual producing a maximum of 42 rings during its lifespan. There was a highly significant positive relationship between the number of rings and the dorsal (r^2 = 0.748, n= 468, P <0.0001) and lateral (r^2 = 0.742, n = 363, P <0.0001) growth of the clam shrimp in the MNJ with the number of rings increasing with size (Figure 16).

Molts

On average, individuals reared in the laboratory molted every 2.59 days with a maximum of 19 days in between molts. Individuals collected from the field molted every 1.3 days with a maximum of 10 days in between molts and producing up to 21 molts during their lifespan. Some individuals never molted (Table 3). The number of days between molts was consistent as an individual increased with age. (r^2 =0.1028, P = 0.0056) (Figure 17).

However, the number of times an individual molted and what body part molted varied. Some individuals molted only the exoskeleton. Some individuals molted the exoskeleton and the full carapace. Some individuals molted the exoskeleton and the outer rings of the carapace. Some individuals molted the outer rings of the carapace alone and some molted the only the full carapace at a time. Each body molt took place when the clam shrimp reached a certain size ($F = 3.06$, $P = 0.0004$).

Age at Maturity and Egg Production

Only 2 of the 61 larvae, reared in the laboratory, reached maturity. Each took 21 and 32 days of age (respectively) to reach maturity. An average of 10.90 eggs/day was produced with a maximum of 143 eggs. In both cases, eggs were produced and deposited over a 7 day period after maturity was reached, with no eggs produced after.

Longevity

Average lifespan for *C. gynecia* after hatching was 13.92 ± 8.03 days, with the earliest death being recorded on day 2, and no shrimp surviving beyond 48 days. Individuals collected from the field survived on average 25.78 ± 17.01 days in the laboratory with the earliest death being recorded 4 days after collection and no shrimp surviving past 89 days from date of collection.

DISCUSSION

Different life histories are experienced among the different orders of branchiopods. Cladocerans are the only branchiopods that are found in large ponds, lakes and

streams and as a result undergo direct development. Cladoceran eggs develop into embryos in the brood chamber with the similar general appearance of an adult. Growth can only occur as they go through successive molts. (McLaughlin 1980; Schram 1986; Thorp and Covich 1991).

Anostracans, notostracans and conchostracans are found in habitats without any currents that could sweep away the larvae. These branchiopods begin life as nauplii or metanauplii. Notostracan sp. nauplii/metanauplii undergo a dozen or more molts before maturity with the adults molting continuously through life. Anostracan nauplii emerge from resting eggs. They undergo a series of molts in which segments and appendages are added, before gradually developing into adults. Adults also continue to molt throughout life. Anostracans are sexually dimorphic and fertilization through insemination occurs after an adult female molts. Conchostracans hatch from resting eggs as well. A carapace is usually not seen until the third naupliar stage (Thorp and Covich 1991). As for the previous two orders, conchostracans go through a series of successive molts until the immatures gradually come to resemble adults.

In dioecious species of clam shrimp, copulation only occurs during a short period of time after a female molts, a pattern found in most arthropods (Goodenough et al. 2010). *Eulimnadia texana* and *Limnadia badia* are two clam shrimp found to follow and guard pre-molt females to prevent them from mating with others. They also follow newly molted females as they await a chance to mate. Males may also attach themselves physically to their potential mates for hours to days at a time (Weeks and

Benvenuto 2008). Females that are carrying eggs within their carapace release their eggs during or immediately before molting, which is a sign that she is ready to be mated (Benvenuto et al. 2009). There is also a possibility that an ecdysteroid (molting hormone) is released during molting and is used as a chemical signal to indicate receptivity (Goodenough et al. 2010).

Most large branchiopods start to hatch within the first 4 days of inundation (Waterkyn 2009, Weeks et al. 1997). *Caenestheriella gynecia* started to hatch 5 days after the soil was rehydrated. The rate and time to complete hatching differed among populations with the MNJ population having the most onsets of hatching, hatching at least three different times during their hatching period (Figure 14). This suggests that the LPMA and SNY populations are homogenous, while MNJ populations consist of heterogenous lineages. None of the individuals reared in the lab acquired the bright orange-red to maroon color typical of *C. gynecia*. This may be a result of high levels of dissolved oxygen within their individual jars. Martinez-Pantoja et al. (2002) found that *E. digueti* and *Leptestheria compleximanus* acquired their bright red color when the ponds lost water and the organisms became overcrowded. Dexter and Ferguson (1943) observed similar color changes in the fairy shrimp, *Eubranchipus serratus*. Color changes occurred as the fairy shrimp grew taking on several hues from light gray with slight traces of orange, brilliant greens, brown, or reddish brown color. They hypothesized that there some relation between the colors of the fairy shrimps and the characteristics of the pond in which they were found. Similar circumstances could explain the lack of color in lab-reared individuals as their environment

consisted of clear aged tap water. The red color of *C. gynecia* collected in the field would therefore most likely be the result of the light reddish clay soil found on the dirt road that may have originated from nearby red Triassic shale and sandstone (Schmidt and Kiviat 2007). This hypothesis would also explain why LPMA and SNY clam shrimp was slightly green in color rather than red as the soil found in the puddles here were a grayish-green color. Color change studies can be done in the future to see if larvae grown in water with soil from the field change color as it grew as compared to larva grown in aged taped water.

Mattox and Velardo (1950) found that it requires 23 days for *C. gynecia* to complete its life history (from egg to maturity). In this study, it took an average of 27 days for the two individuals to reach maturity. A total of 143 eggs were produced during their lifespan. Four individuals lived 22 days and did not reach maturity, indicating that 23 days may be the developmental threshold for *C. gynecia.*

Some of the hatchlings from the experiment died quickly after isolation. A similar situation occurred when Day et al. (2010) performed rearing experiments of some of the branchiopods found in Western Cape, South Africa. They noticed that hatchlings from eggs cultured in sediment survived longer than those that hatched from isolated eggs. A probable cause may result from the inability of the nauplii to acquire food through filtration. The early larval stage is characterized by three pairs of appendages (attenules, antennae and mandibles) which are primarily used for locomotion. It is not until post- naupliar development, where there are additional limbs that the larva is

able to filter food from the water column (Schram 1986, Barrington 1979). Administration of artificial food (i.e., an algal culture or yeast) proved to be futile. The sediment in which the eggs were collected would contain the propagules of natural algal and fungal foods and would be sufficient nutrition for the hatchlings (Day et al. 2010). I recommend that water (filtered for eggs) from the tank should be used in future experiments to replenish necessary nutrients found because of the natural mixing of the soil and water.

Daily growth and Molts

Caenestheriella gynecia larvae grew 0.143 mm/day dorsally. Individuals collected from the field grew 0.052 mm/day dorsally. Individuals collected from the field that produced eggs grew on average 0.142 mm/day. Weeks et al. (1997) found that there is a trade-off between growth and reproduction in *E. texana.* Before the onset of maturity, growth increments are very high but start to decline to near zero as egg production starts. Similar conclusions can be drawn for *C. gynecia* as most eggs were produced during growth increments of 0.00 – 0.05 mm/day (Figure 19). This is likely the result of the reallocation of energy to egg production versus growth.

In crustaceans, the carapace is a two-walled structure that can be separated into an outer and an inner wall lined by epidermis cells. The tough outer wall is composed of a "hard" cuticle that can be calcified in some orders of crustaceans. The inner wall has a much thinner cuticle. Blood is circulated in between these walls and is involved in respiration (Fryer 1996). In order for growth to occur, crustaceans molt and the carapace must be shed and replaced by a new and larger one.

Fryer (1996) reviewed the origin of the crustacean carapace within several orders of branchiopods. He described in detail the development of the conchostracan carapace in which the carapace arises from a fold of the integument (covering) from the posterior margin of the cephalic region and extends by the secretion of new cuticle beneath the old. The carapace is attached to the body by a pedicel without any attachment to the thoracic segments. The paper was produced in rebuttal to Walossek's (1993) proposition of a thoracic origin in which the carapace originated and is fused to the thoracic appendages of crustaceans without the appearance of a fold at any stage of growth.

Three types of molts were observed during the experiment: exoskeleton, full carapace (both valves), and the outer rings of the carapace. There are currently no records of this type of molting in branchiopods. Most studies have reported the number of times the organism molts but does not detail which part molts. Obregón-Barboza et al. (2001) only observed the number of days between each molt in the tadpole shrimp *Triops*. Weeks et al. (1997) looked at the number of molts per day in the clam shrimp *Eulimandia texana* to see if there was a correlation between molting and growth rate or daily egg production. They found that molting was more correlated to egg production than to a significant increase in size. It is assumed that they are referring to the exoskeleton alone. Olesen (2005) studied the molting of the larvae of the clam shrimp, *Lynceus brachyurus*, during the development of the carapace from the naupliar dorsal shield. In his study the larva molt consisted of its exoskeleton attached to its naupliar shield. The author does not make any observations on the

molts made after the last nauplius stage. The described molting pattern of *Caenestheriella gynecia'*s is the first report of molting data supporting the classical view of cephalic origin of the carapace in branchiopods. If the carapace of *C. gynecia* was fused to the thoracic appendages, all molts collected would possess the exoskeleton of the body of the clam shrimp and the carapace in one molt. This was not observed at any molt collection during this study.

Several factors need to be taken into consideration when interpreting the results of this study. As stated in the methods section, "immature" clam shrimp were collected in the field and they were considered immature if no eggs or claspers were visible. However, this may affect the data on the life history as unknown number of life history measures may have occurred already in the field (i.e., an individual may have molted six times in the field before collection). Nonetheless, the data from this study can provide a base of information that can be useful in further examinations of the biology and ecology of *C. gynecia*.

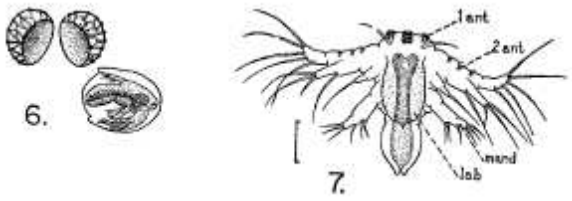


Figure 12. *C. gynecia* nauplius hatching from egg (6); *C. gynecia* nauplius with 1st antennae buds and 2nd antennae used as swimming organs (Mattox 1950).

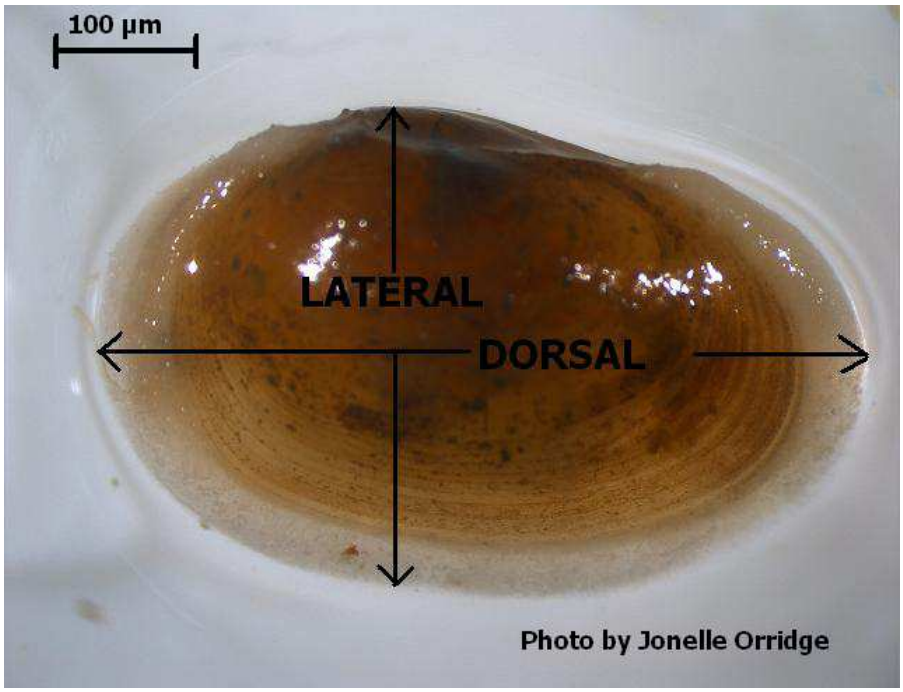


Figure 13. Dorsal (measured between the two ends of the elliptical shell) and lateral (measured from the umbone of the carapace to the opposite end of the shell) measurements.

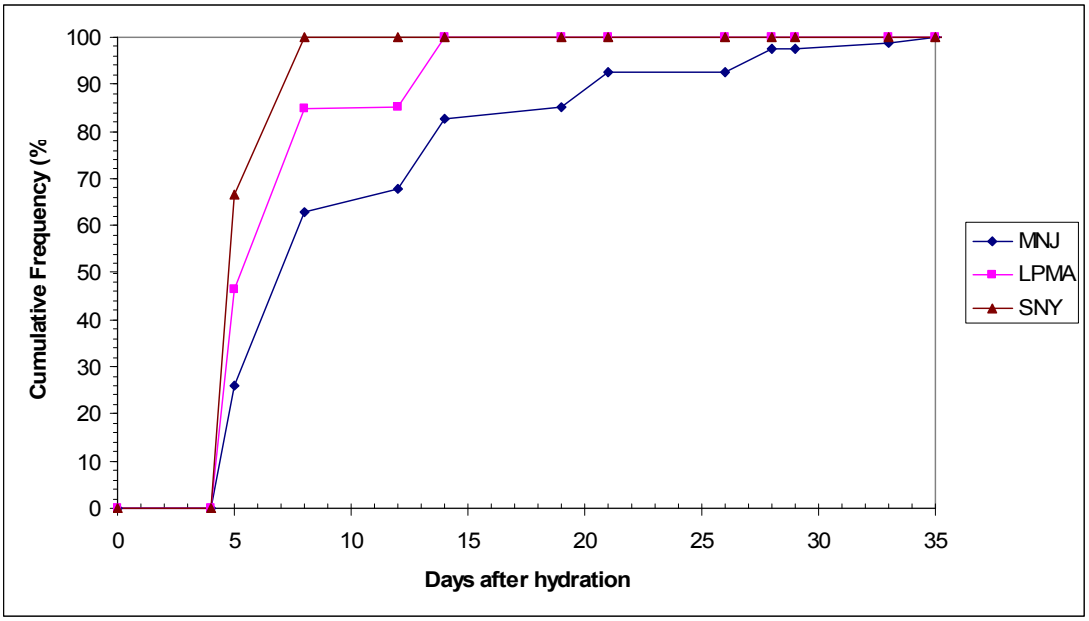


Figure 14. Comparison of hatching times of *C. gynecia*. Onset of hatching began 5 days after hydration for all populations. The point at which 50% and 100% of the larva hatched differed for each population.

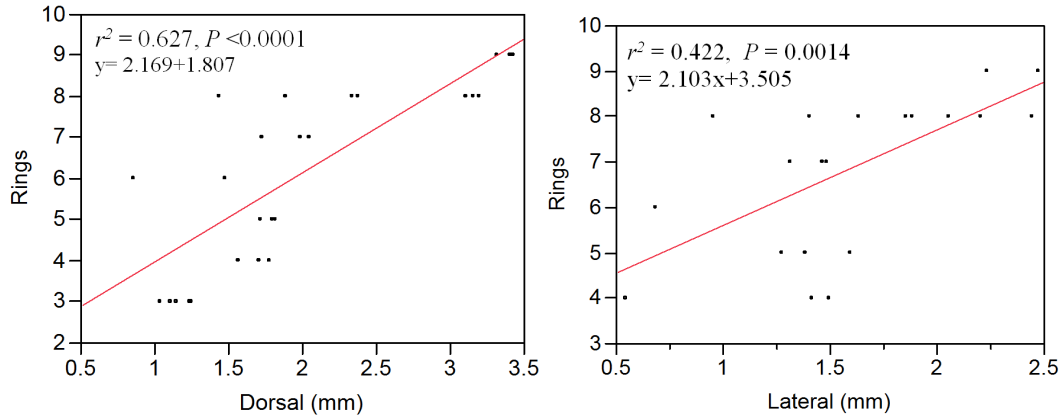


Figure 15. Relationships between dorsal (n = 30) and lateral (n = 22) measurements and number of rings present on the carapace of *C. gynecia* reared in the laboratory.

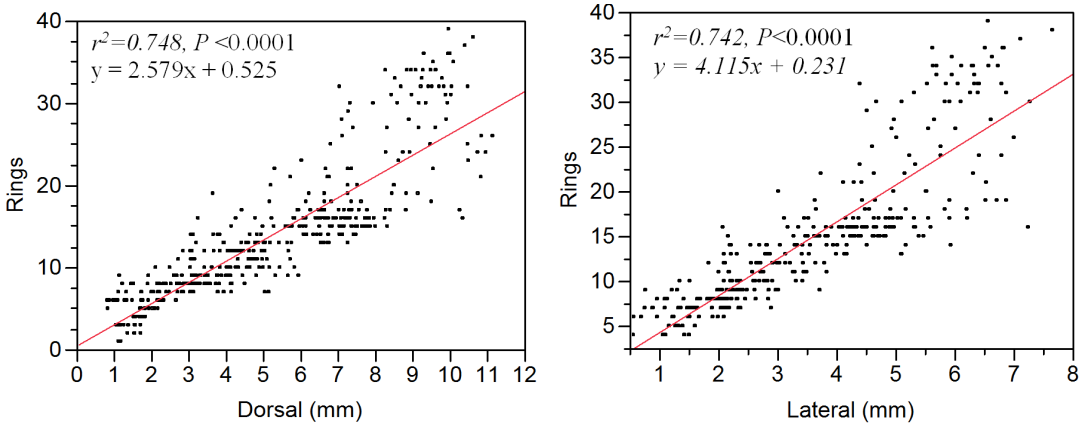


Figure 16. Relationships between dorsal (n = 467) and lateral (n = 363) measurements and the number of rings present on the carapace of the MNJ population of *C. gynecia* caught in the field during 2006-2009.

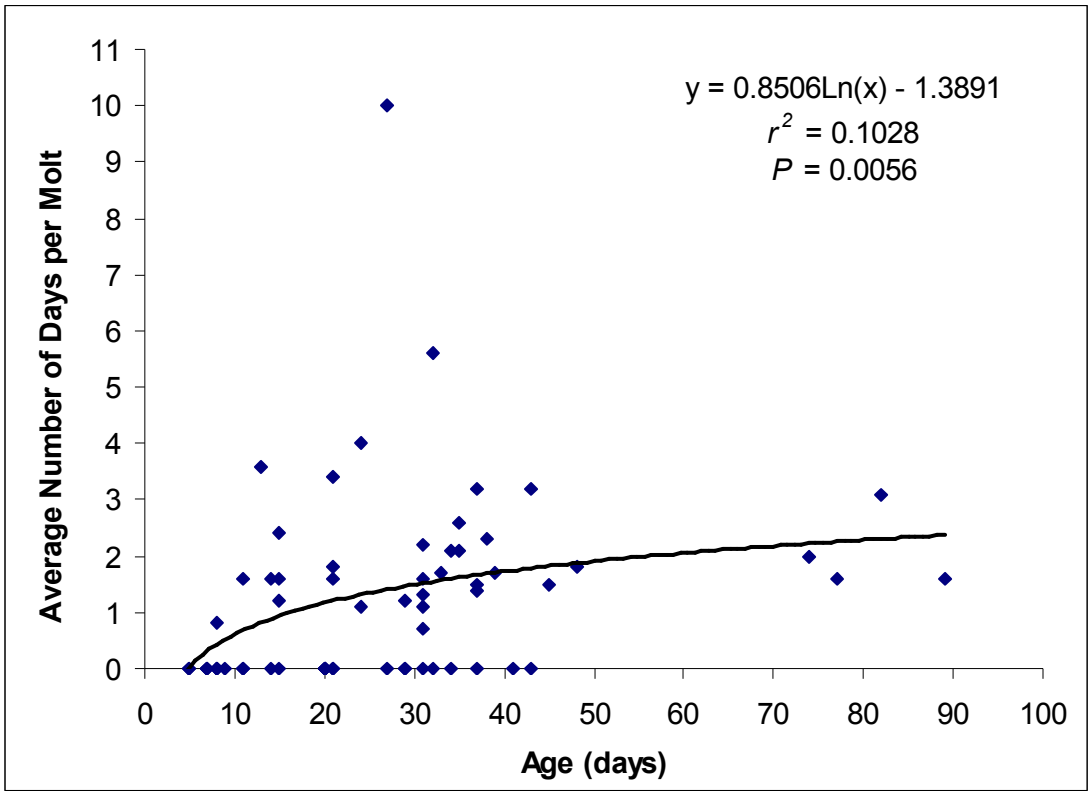


Figure 17. Relationship between the average number of days between molts and the age of field caught *C. gynecia* (n = 76).

Table 3. Life history characteristics of *C. gynecia*. Values in parentheses indicate the range.

Sample group	Dorsal growth (mm/day)	Lateral growth (mm/day)	Molts (days/ molt)	Age at maturity (days)	Daily egg count (eggs/day)
Larvae	0.145 (-0.003 - 0.533)	0.097 (-0.013 - 0.405)	2.59 (0 - 19.2)	26.5 -	10.90 (0 - 143)
MNJ popul-Ation	0.052 (-0.338 - 0.606)	0.041 (-0.244 - 0.675)	1.3 (0 - 10)	14.2 (1 - 31)	11.30 (-9.26 - 67.50)

Table 3 (continued).

Sample group	Long-evity (days)	Ring count (rings/day)	Maximum egg count
Larvae	13.92 (2-48)	0.210 (0 - 20)	143
MNJ popul-ation	25.78 (4-89)	0.40 (0-1.9)	917

CHAPTER III: MORPHOLOGY AND TAXONOMY OF NORTHEASTERN CONCHOSTRACANS

INTRODUCTION

Conchostracan morphology

Clam shrimp possess a two-part carapace similar to that of a bivalve mollusk. It is laterally compressed and typically encloses the entire body. The carapace is joined to the body by a ligament and is held together by a pair of strong adductor muscles in the cephalic region. The clam shrimp reacts to danger by contracting these muscles, so that the valves close tightly while the organism lies motionlessly at the bottom of the pool. The carapace is marked by concentric growth lines and the presence of an umbone – the dorsal-most part of the carapace that represents the early growth stage of the shell (Schram 1986, Pearse 1987, Thorp and Covich 1991, Stachowitsch 1992). Several theories were proposed about the origin of the carapace and whether it consists of fused tergites (the longitudinal, dorsal surface of a body segment of most arthropods) (McLaughlin1980) or develops via a cephalic fold. Fryer (1996) has since shown that the latter occurs as the carapace develops when new cuticle is secreted beneath the old one by the underlying epidermis and this is repeated at each molt throughout life. The carapaces of clam shrimp belonging to the *Caenestheriella* and *Cyzicus* genera, both found in the northeastern U.S., are similar (Figures 18 and 19).

The sessile compound eyes of clam shrimp are close together and are located above the rostrum. The first pair of antennae is reduced and unsegmented. The second pair

of antennae, however, is long and biramous and both branches are covered with numerous bristles. A discordant number of segments may be found between the two pairs of antennae due to independent development during ontogeny (Maruzzo et al. 2009). Members of the conchostracan suborder of crustaceans swim primarily by moving the antennae and the thoracic legs in an oar-like manner (McLaughlin 1980). The number of segments constituting the thorax varies from 10 to 32, with one pair of legs per segment (Figure 20).

Arthropod limbs are morphologically diverse and serve as one of the key diagnostic and evolutionary features in crustacean diversification. They are also a major focus of biologists who study the developmental basis of evolutionary variability in limb morphology. Branchiopod limbs are the most primitive and unique of the crustacea. They are composed of phyllopodous elements resembling flattened paddles that vary in the number of proximal lobes on the medial and lateral margins (Williams 2007). In females, the outer lobes of several middle legs are modified into long, threadlike outgrowths that are directed dorsally and used to hold the eggs on the dorsal side of the body under the carapace (Figure 21). However, the main functions of the thoracic legs are to carry food forward to the mouth and for respiration. The outer lobes of all thoracic legs serve as gills. In order to carry out these functions, the legs are in constant movement, and the water between the valves of the carapace is quickly renewed. The combination of carapace circulation and the fleshy trunk limbs is the principal locus for respiration in the clam shrimp. In males the first pair of trunk limbs lose the phyllopodous lobes and are modified to claspers used in attaching

themselves to the females during copulation. The body ends in a large chitinized telson that is either laterally compressed and bears a pair of large hooks, or is dorsoventrally compressed, with short hooks (McLaughlin 1980, Schram 1986). Classification of *C. gynecia* is difficult due to the characteristics that separate it from its nearest relative. Using the *Taxonomic Key to Genera of Non-cladoceran Freshwater Branchiopoda* (Mattox 1957), it is possible to identify conchostracans to the genus level. *Caenestheriella gynecia* is distinguished from *Cyzicus* by the difference between the male and female rostrum. In *Cyzicus*, the male rostrum terminates bluntly versus an acute rostrum in the females (Mattox 1957) (Figure 22). Since no males have been found for *C. gynecia,* it is defaulted into the *Caenestheriella* genus based on meristics. The species, *C. gynecia* is defined on the basis that no individuals have been found with male claspers (Thorp and Covich 1991).

Recent populations found in the New Jersey Meadowlands; Sugar Loaf and Hyde Park New York; Lenox and Pittsfield, Massachusetts are thought to be *C. gynecia* on the basis of the absence of males and their ecology (Smith and Gola 2001, Schmidt and Kiviat 2007). In this section, I assess the morphology of *C. gynecia* to determine whether this population is correctly placed in *Caenestheriella*. If males were found, comparative shape of the female and male rostrum with the genus *Cyzicus* might modify classification of the species.

METHODS

Collection

Individuals of *C. gynecia* used for morphological comparisons were collected from the following study sites using a 500-micron dip net: Meadowlands, NY (MNJ); Lenox and Pittsfield, MA (LPMA); and Sugar Loaf, NY (SLNY). Individuals were collected by sweeping the dip net through the ephemeral puddles. Specimens were transported in water from the collection site. In the lab, organisms were rinsed with aged tap water and placed in sterile polystyrene Petri dishes filled with water from the puddles in which they were collected. Live individuals were observed for life history traits (results in chapter II) and when they perished they were preserved in 70% isopropanol.

Morphological comparisons

Two tweezers were used to dissect specimens, one to slightly open the carapace and the other to lift and remove one side of the carapace from the other exposing the body. Several morphological and meristic characteristics of populations of *C. gynecia* were compared after being dissected (Table 4). Carapace size was measured to the nearest 0.01mm using a Mitoyo Dial Caliper. The number of antennal segments, thoracic appendages, and telson spines and rings were counted with the aid of the image analysis software Leica Application Suite v. 2.3.2R1. Males and females were determined by the absence or presence of modified 1st and 2nd pairs of thoracic appendages. If no modifications were found, individuals were scored as females.

Statistical analyses

Variations in each meristic character among *C. gynecia* populations were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test to find which populations are significantly different from one another. Both tests were performed in JMP v. 8.0 statistical software. Results were deemed significant at $P < 0.05$.

RESULTS

Morphometrics of Caenestheriella gynecia

Only females were found among the 363 individuals collected and examined from Meadowlands, NJ (MPNJ); 41 individuals from Lenox and Pittsfield, MA (LPMA); and 16 individuals from Sugar Loaf, NY (SLNY). None possessed any male claspers, either fully or partially developed.

Specimens from MNJ ranged in size from 1.20 mm to 11.54 mm ($\bar{x}$ = 6.08 mm) total dorsal carapace length (Table 5). The carapace growth rings ranged from 7 to 26 ($\bar{x}$ =14, n = 46) with a significant positive correlation between carapace size and ring number ($r^2 = 0.67$, $P < 0.0001$) (Figure 23).

Among MNJ specimens, the rami (branches) of the antennae had between 1 to 23 segments ($\bar{x}$ = 13, n = 189). MNJ specimens possessed 5 to 38 ($\bar{x}$ = 17, n = 261) spinal pairs on the telson and the number of thoracic appendages ranged from 10 to

33 pairs ($\bar{x}$ = 19, n = 212). There was a strong positive relationship between the size of the clam shrimp and the number of right antennal segments (r^2 = 0.51, P <0.0001) and left antennal segments (r^2 = 0.45, P <0.0001) thoracic appendages (r^2 = 0.49, P <0.0001) and telson spines (r^2 =0.31, P <0.0001) with a trend of increasing meristics with size of the carapace (Figure 23 and Table 6).

Morphometric comparisons among populations of Caenestheriella gynecia

Forty-one specimens from LPMA and 16 from SNY were dissected for morphological comparisons with MNJ. Morphological measurements and counts varied among the three populations of *C. gynecia* (Figure 25, Table 5). Individuals from Massachusetts had an antennal segment range between 8 and 19 ($\bar{x}$ = 14, n = 33) and individuals from New York ranged from 13 to 20 ($\bar{x}$ = 17, n = 10). Individuals from Massachusetts possessed 16 to 26 ($\bar{x}$ = 20, n = 33) spinal pairs on the telson and 24 to 38 ($\bar{x}$ =29, n = 12) spinal pairs in the New York collection. Individuals from Massachusetts ranged in size from 4.86 mm to 10.81 mm ($\bar{x}$ = 6.94 mm, n = 41). Individuals from New York ranged in size from 8.98 mm to 13.1 mm ($\bar{x}$ = 10.70 mm, n=16). The number of thoracic appendages ranged from 12 to 24 pairs ($\bar{x}$ = 20, n = 22) in the Massachusetts population and 15 to 24 pairs ($\bar{x}$ =19, n = 10) in the New York population. Growth rings ranged from 11 to 21 ($\bar{x}$ =15, n = 28) in the Massachusetts population and 25 to 41 ($\bar{x}$ =34, n = 16) in the New York population (Figure 24 and Table 5).

Average size differed significantly among populations of *C. gynecia*. Specimens from SLNY were larger than those from LPMA and MNJ populations (P <0.0001).

Specimens from LPMA were larger than MNJ populations ($P < 0.0001$). Data were separated and analyzed by month of collection to remove potential ecological artifacts (i.e., different size clam shrimp found at different times in a growing season). During the month of July, clam shrimp from the LPMA population were significantly larger than clam shrimp collected in MNJ ($P <0.0001$) and SLNY were significantly larger than clam shrimp from MNJ collected during the month of September ($P <0.0001$) (Table 7).

A significant difference in the average number of antennal segments was observed between SLNY and the MNJ and LPMA populations ($P = 0.0092$ and $P = 0.0326$, respectively) with SLNY possessing a greater number of right and left antennal segments than the other two. There was a significant difference in the number of left antennal segments between the SLNY and MNJ with SLNY possessing more segments ($P = 0.0286$) (Table 6). There was no significant difference in the number of right antennal segments ($P = 0.9685$) and left antennal segments ($P = 0.9663$) between MNJ and LPMA. Separation of data by month revealed that there was only a significant difference between SLNY and MNJ in the average number of right antennal segments in the month of September ($P = 0.039$) and no difference in the amount left antennal segments ($P = 0.353$) (Table 7).

The average number of spinal pairs found on the telson was significantly different among all three populations with SLNY ($P <0.0001$) having the largest number of spinal pairs followed by LPMA ($P = 0.0462$). MNJ ($P = 0.0462$) had the fewest spinal

pairs on the telson (Table 7). Separation of data by month revealed that that SLNY had significantly more spinal pairs than MNJ (P <0.0001) in September and LPMA had significantly more spinal pairs than MNJ (P <0.0001) in July (Table 7).

Growth rings found on the carapace differed significantly among the three populations. The average number of SLNY growth rings (P <0.0001) was significantly higher than both LPMA and MNJ. LPMA had more growth rings than MNJ (P = 0.0097). When data were separated by month of collection, SLNY (P = 0.0144) had significantly more rings than MNJ during the month of September (Table 7).

No differences in the amount of thoracic appendages were found among the three populations of *C. gynecia* except when separated by month. SLNY had significantly more thoracic legs than MNJ (P = 0.0126) when collected in September.

DISCUSSION

Classification of Caenestheriella gynecia

Classifying branchiopods has been difficult because of overlapping ranges in their distribution and morphology (Table 9), and the variability in the characters used to diagnose a species (Smith and Wier 1999). There has been speculation as to whether *C. gynecia* has been wrongly placed within *Caenestheriella* and perhaps should be included within *Cyzicus* (Smith and Gola 2001). This hypothesis was based solely on

morphological comparisons and the fact that there have been no males recorded for *C. gynecia* (Smith and Gola 2001). All 404 individuals dissected for morphological comparisons in this study were found to be female, by morphological criteria, independent of the population analyzed.

Although males were not found in this study, future studies of *C. gynecia* could reveal the presence of these rare males if they do exist. It was discovered that a once thought to be strictly parthenogenetic species of clam shrimp, *Limnadia lenticularis*, actually possessed some males within a population. Eder et al. (2000) reinvestigated all conchostracans collected and deposited at the Naturhistorisches Museum Wien in Austria, between 1994 and 1997, and found that 4 of the total 364 individuals available were males. The new records of these males disprove the previous notion of an exclusive parthenogenetic mating system of *L. lenticularis,* but the authors point out that these are rare exceptions and do not constitute normal male:female ratios of clam shrimp with an androdioecious or dioecious mating system. Future studies on *C. gynecia* could reveal similar results if extensive sampling of this species occurs.

Morphological comparisons among populations of Caenestheriella gynecia

Although wide in range, the mean values of each meristic feature are actually close, if not the same, between the NJ and MA populations (Table 7). All values are within those described by Smith and Gola (2001) of the original population discovered by Mattox and Velardo in 1950 and also their sample population (n =186) in Massachusetts (Figure 25). Individuals (n = 11) collected by Schmidt and Kiviat

(2007) from the same MNJ site also had measurements which fell into the same range as those examined in this study.

Individuals collected from the NY populations were larger, at time of collection, than those in NJ and MA. This may be due to longer pond duration of the puddles found in NY, where puddles are heavily shaded by trees and are not subject to high levels of evaporation due to direct sunlight and significantly cooler temperatures than NJ ($P<$ 0.001). In addition, larvae can still be present in the field in NY until late November (Jay Westerveld, New York Natural History Council, personal communication). Pond duration constrains the length of life of a branchiopod. Clam shrimp living in ponds of short duration should have a faster growth rate than those in ponds with longer duration because a minimum size must be met before they can be reproductively mature. Size and age at maturity varies among species of clam shrimp and populations. *Eulimnadia texana* and *E. diversa* can reach maturity between 4 and 11 days of life (3 to 4 mm long) while *C. gynecia* takes approximately 23 days to reach maturity, ranging in size from 7 to 11 mm (Weeks et al. 1997, Mattox and Velardo 1950, Emberton 1980, Thorp and Covich 1991). Differences in pond duration among populations would allow the clam shrimp in NY more time to grow as long as other habitat conditions were still favorable (i.e., temperature, food availability, etc.) (Marcus and Weeks 1997). This would account for the larger individuals found in NY when compared to MA and NJ, whose puddles frequently evaporate and refill during a season because of the puddles are exposed to direct sunlight, not shaded by surrounding foliage.

During clam shrimp ontogeny, there is a distinctive pattern of the addition of segments and limbs through successive stages starting as a free-living larva until adulthood. The number of stages range from six to ten (or more) depending on the species (Table 9). There is one spinicaudatan that differs from this general pattern, *Cyclestheria hislopi. Cyclestheria hislopi* has direct development of embryos within its carapace and does not have any free-living larvae. Larvae undergo their first four stages of morphological differentiation in the protection of their 'mothers' carapace and emerge as juveniles (Olesen 1999). Pabst and Richter (2004) compared the larval development of seven species of clam shrimp *(Limnadopsis parvispinus, Limnadia stanleyana, Eulimnadia texana, Imnadia yeyetta, Eulimnadia braueriana, Limnadia lenticularis and Caenestheriella packardi)* and discovered that there was a difference between the degree of limb development and carapace size. Different species had different times of appearance of additional appendages at different carapace sizes. In this study, this distinction between limb number and size was evident among the three populations (Tables 6 and 7). Future comparitive studies should be performed on the larval development between NJ and NY populations of *C. gynecia* and *Cyzicus* species as it is possible to identify a species by its unique combination of successive morphological characters during ontogeny (Olesen 1999, Olesen and Grygier 2003, Pabst and Richter 2004).

Another discordant arrangement of segments was seen in this study where there was a difference in the number of segments found on the right and left antennas of the clam shrimp. This developmental difference is known as segmental mismatch and occurs

when the segmental series of different structures along the same axis show discordant arrangement. In the Maruzzo et al. (2009) study on the antennal exopod of *Artemia*, they found that different structures of the same naupliar appendage can develop as independent segmental units. The results implied that there is a degree of independence of developmental pathways in the two sides of the appendage during embryogenesis. This 'uneven' development can be maintained until late in development. This occurrence is not rare in arthropods and is also observed in other branchiopods. Linder (1952) found differences in the length of the series and postembryonic segmentation schedule among dorsal and ventral structures in notostracans.

Clam shrimp from SLNY were larger and had more thoracic appendages than those from the MNJ population, in addition to possessing more antennal segments, spinal pairs, and number of growth rings. New York populations fell outside the *Caenestheriella* range (21-35 rings) for the number of growth rings with most individuals possessing up to 41 growth rings. They also fell outside the *Caenestheriella* range (~15) for the number of telson spines, possessing 29 pairs on average. This comes closer to the expected maximium number of telson pairs for a *Cyzicus* species as they can possess approximately 31 pairs (Table 8).

With these results, I conclude that the MNJ and LPMA clam shrimp are correctly diagnosed as *Caenestheriella gynecia* and I recommend that the clam shrimp found in NY should be placed in the *Cyzicus* genus. The closest *Cyzicus* species whose range

overlaps with *C. gynecia*'s is *Cyzicus mexicanus* and *Caenestheriella setosa* (Mattox 1957, Schmidt and Kiviat 2007). As dispersal of clam shrimp is mediated by the aid of wind and human and animal vectors, it is possible that *C. mexicanus* or *C. setosa* was transported to NY by dispersal events which occurred after the Wisconsinan Glacial episode. Geographical parthenogenesis may have occurred within the species due to geographic barriers which forced them to colonize the resulting marginal habitats (Kearney 2005, Vorburger 2006, Schön 2007).

It was noted by Smith and Gola (2001) that populations of *C. gynecia* were first found in shallow muddy pools formed by the tires of vehicles along wooded roads, sometimes found adjacent to railroad grades in 1939. In the late 1820s, the Baltimore and Ohio Railroad started operation and served New York City; New York via Philadelphia, Pennsylvania; Baltimore, Maryland, and Washington, D.C. to Chicago, Illinois and St. Louis, Missouri (Frasca 2010). It is possible that the construction and daily use of the train system allowed *C. gynecia* and/or *C. mexicanus* to be dispersed to the eastern states as the speed of the trains kicked up dust as it traveled on the tracks (Figures 26 and 27).

Another hypothesis is that interstate ATV clubs may be helping to disperse clam shrimp eggs. Through personal communications with ATV owners who frequented the Meadowlands site, ATV clubs travel to different states to 'ride.' When they return to their home state, it is possible that they are transporting clam shrimp eggs within the tire treads. This is supported by the fact that I was able to successfully collect *C.*

gynecia larvae after the rehydration of soil scraped from treads of ATV after a ride down the Meadowlands site.

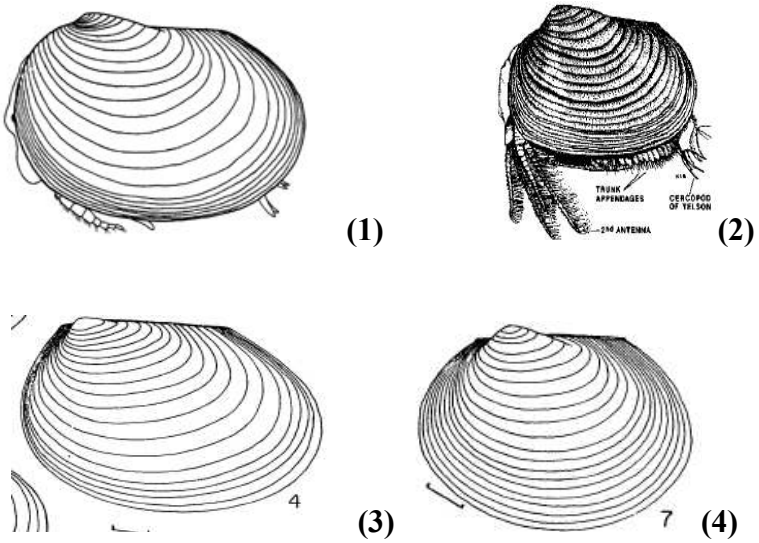


Figure 18. Carapace comparisons of North American *Caenestheriella* genus. (1) *Caenestheriella sp.* carapace. (2) *C. gynecia* adult female. (3) *C. setosa*. (4) *C. belfragei* (Mattox 1957, McLaughlin 1980, Emberton 1980).

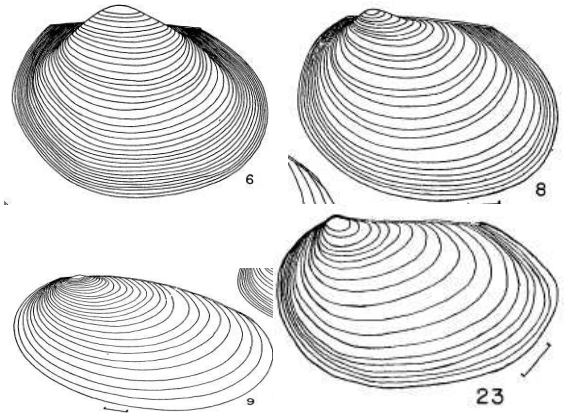


Figure 19. Carapace comparisons of North America *Cyzicus* genus. (6) *C. morsei; (8) C. mexicanus;* (9) *C. californicus;* (23) *C. elongatus* (Mattox 1957).

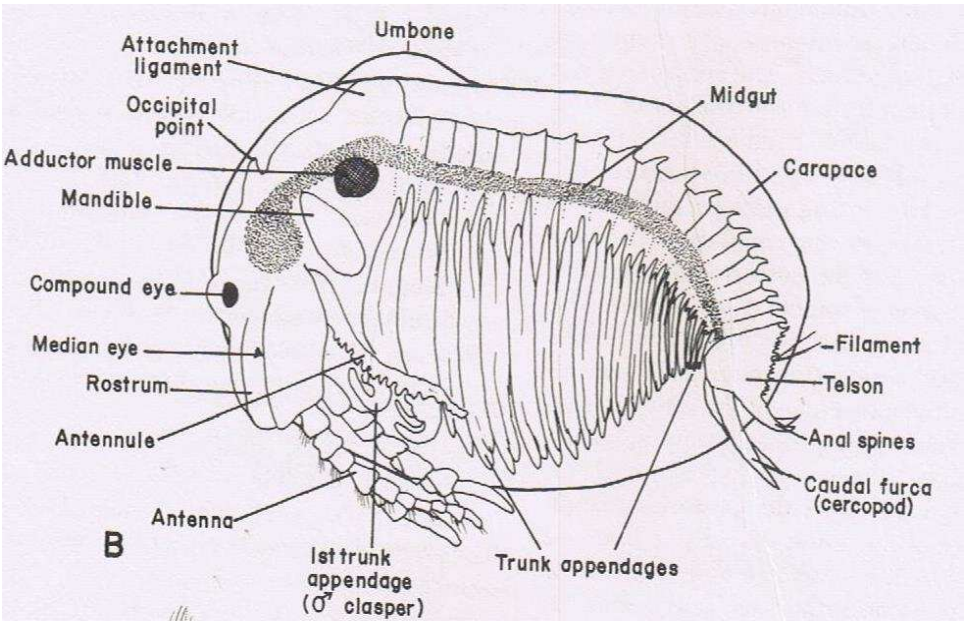


Figure 20. *Caenestheriella* sp. morphology (McLaughlin 1980).

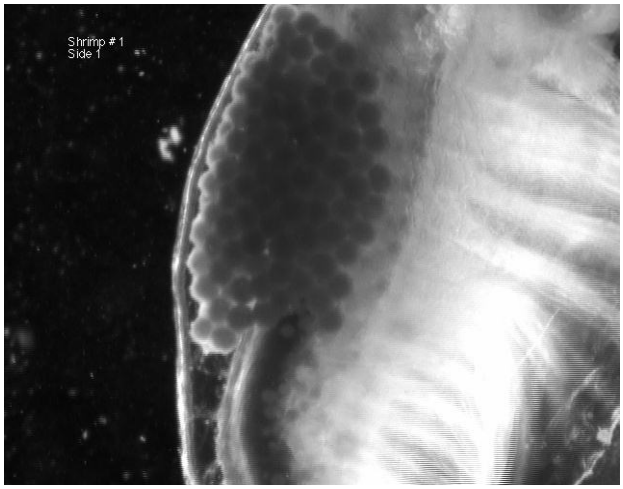


Figure 21. Eggs underneath the carapace in *Eulimnadia texana* (Weeks, www.clamshrimp.com)

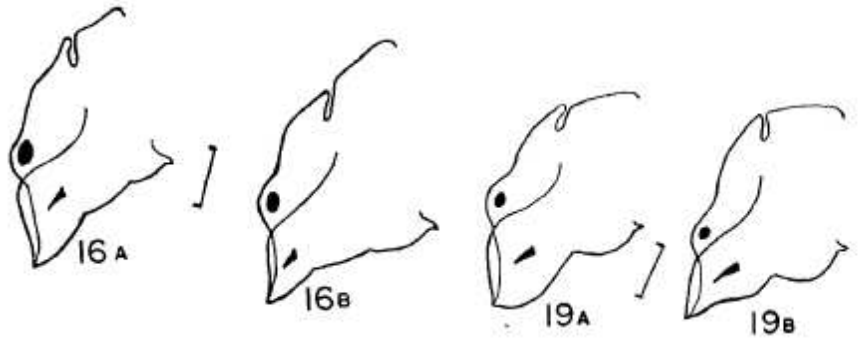


Figure 22. Comparisons of male and female rostrums illustrating the blunt (16a and 19a) vs. acute (16b and 19b) rostrum forms. 16a and 16b are a male and female (respectively) from *Caenestheriella setosa*. 19a and 19b are a male and female (respectively) from *Cyzicus mexicanus*.

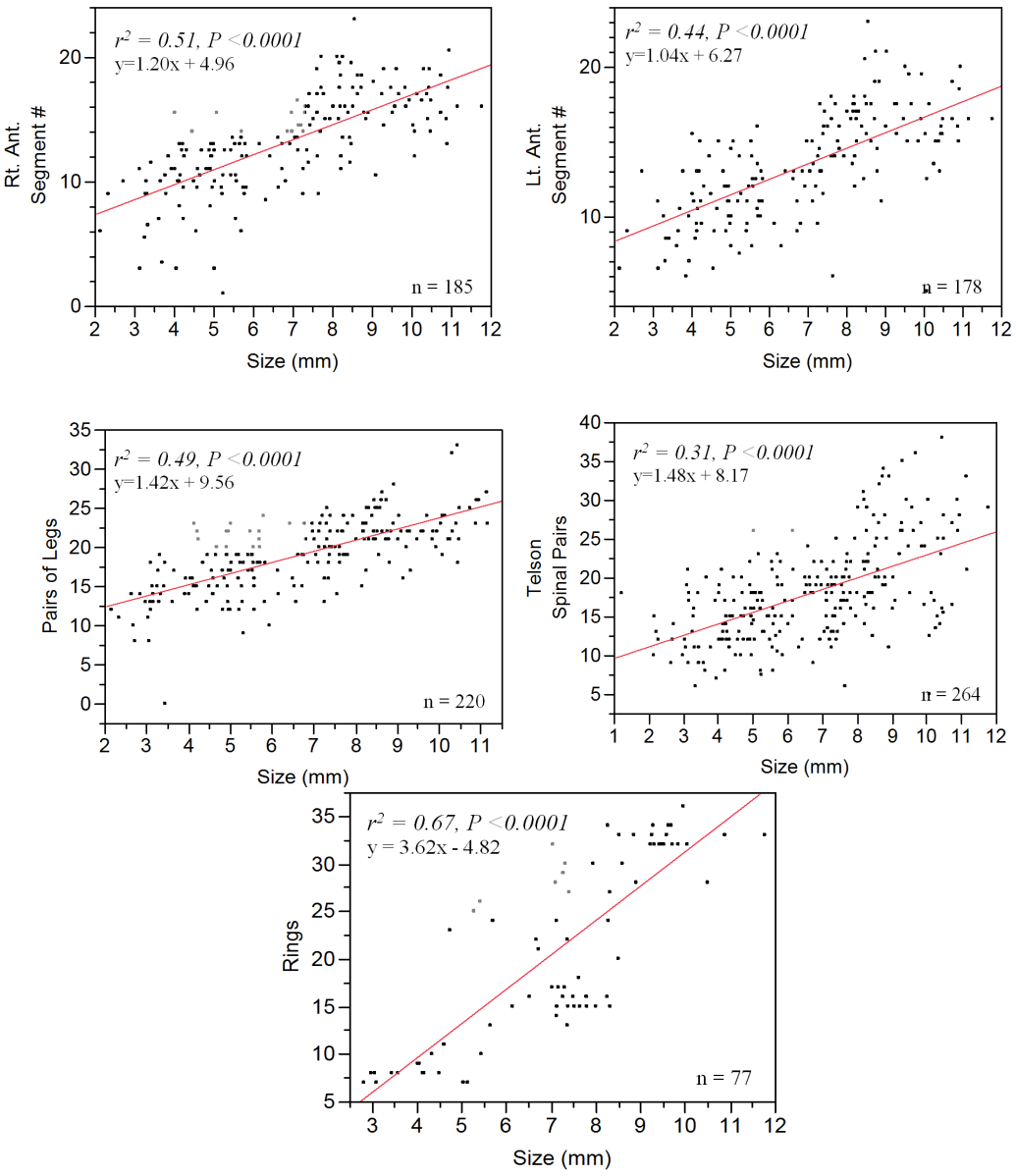


Figure 23. Relationships between size (mm) of *C. gynecia* (MNJ population) and meristic characters. Solid line depicts the least-squares fit of the two variables.

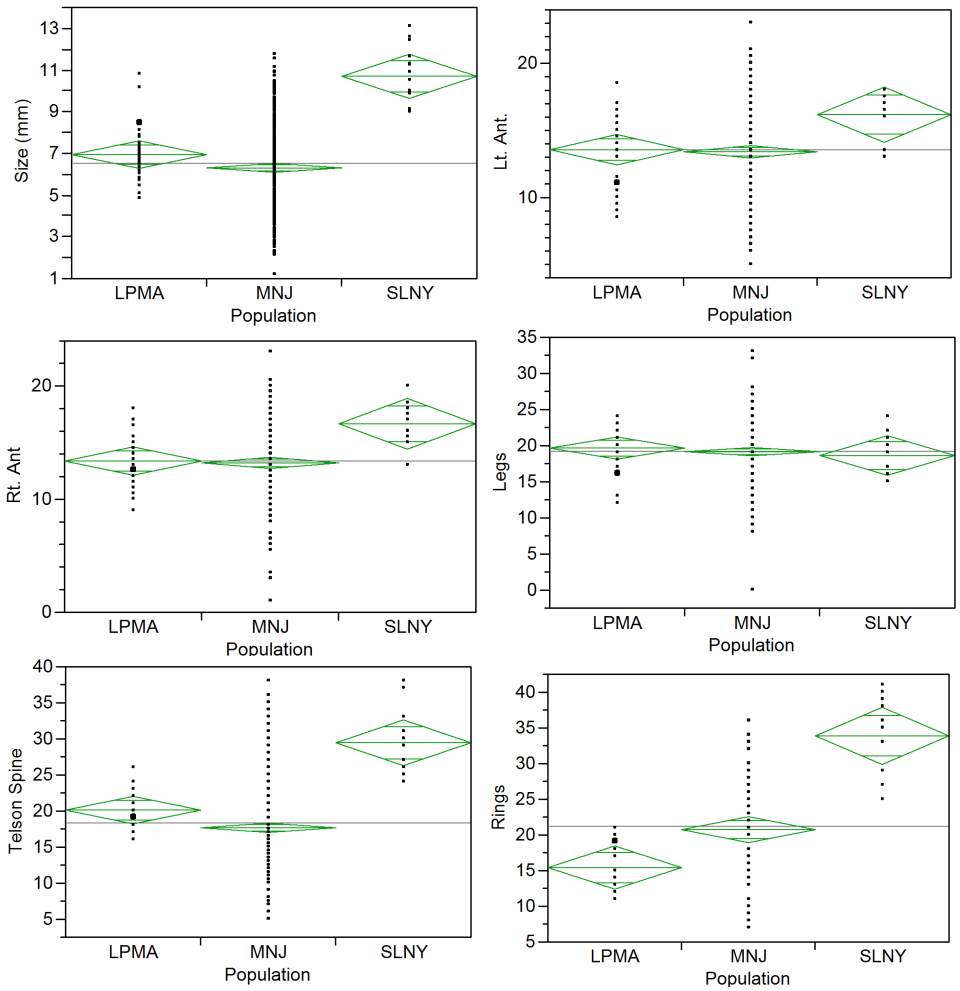
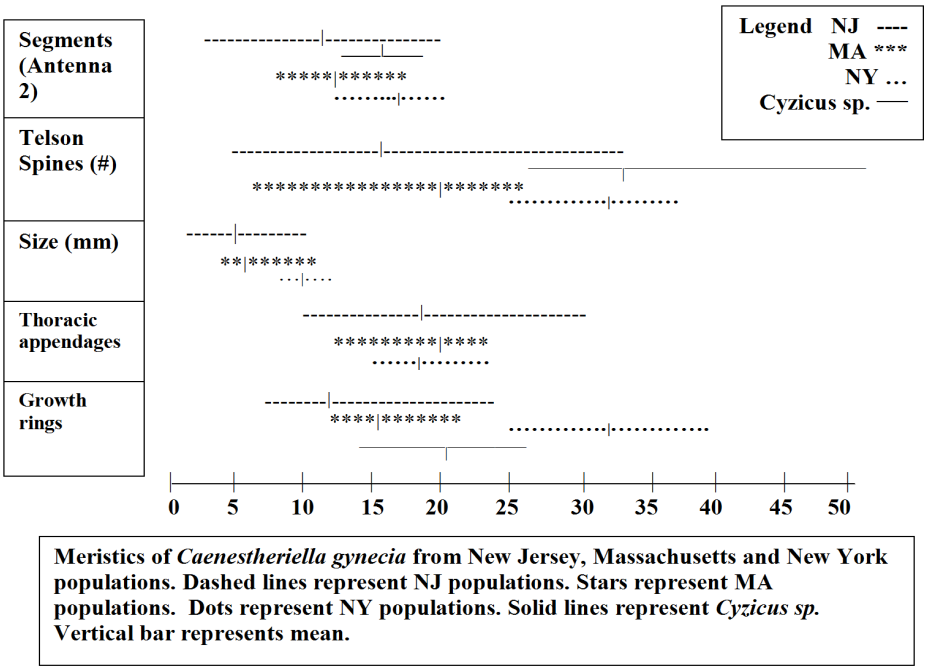


Figure 24. Comparison of meristics among Massachusetts and New Jersey populations of *Caenestheriella gynecia.* Rt. ant: Right antenna segment. Lt. ant: Left antenna segment. (Diamonds display the mean, standard error, upper and lower 95%.)



Meristics of *Caenestheriella gynecia* from New Jersey, Massachusetts and New York populations. Dashed lines represent NJ populations. Stars represent MA populations. Dots represent NY populations. Solid lines represent *Cyzicus sp.* Vertical bar represents mean.

Figure 25. Summary of meristics among *Caenestheriella gynecia* populations.

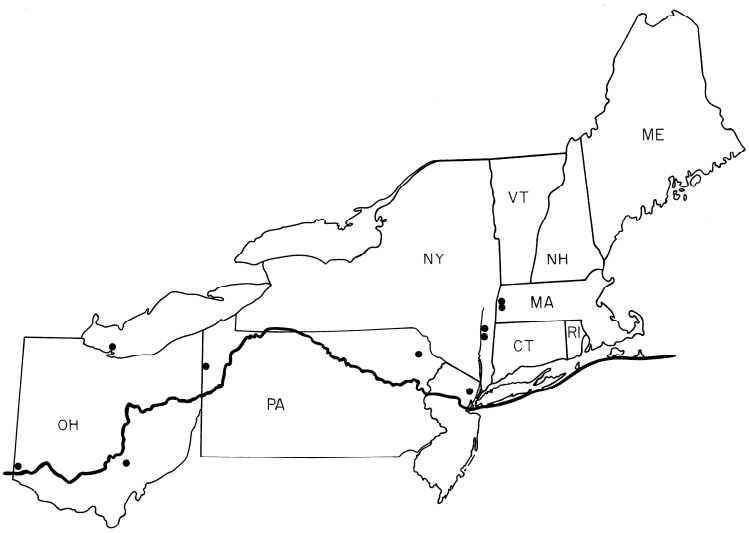


Figure 26. **Map of *C. gynecia* distribution in the eastern United States. The solid line represents the extent of the most recent glaciation (Excerpted from Schmidt and Kiviat 2007).**

Figure 27. **Map of the Baltimore and Ohio Railroad route (http://www.personal.psu.edu/jun3/blogs/pa_center_for_the_book_workshop/americas-first-steam-engine-final-draft/)**

Table 4. Morphological characters assessed.

Morphometric and Meristic Characters	
Carapace size	Range: 5 – 17 mm
Antennae	Biramous; each ramus variously segmented according to taxa
Thoracic appendages	10 – 32 pairs of trunk appendages
Telson	Broad, truncate, with pair of claw-like caudal rami or cercopods; presence of spines
Rings	Consecutive growth lines or rings on the carapace
Sexual characters	Males possess modified 1st and/or 2nd postcephalic appendages

Table 5. Comparison of meristic characters among populations of *Caenestheriella gynecia.* Abbreviations: MNJ: Meadowlands, NJ; LPMA: Lenox and Pittsfield, MA; SLNY: Sugar Loaf, NY; Rt. Ant: Right Antenna; Lt. Ant: Left Antenna; Thor. App.: Thoracic appendages; S.D. (standard deviation).

	MNJ					LPMA					SLNY				
	Mean	**S.D.**	**Min.**	**Max.**	**N**	**Mean**	**S.D.**	**Min.**	**Max.**	**N**	**Mean**	**S.D.**	**Min.**	**Max.**	**N**
Size (mm)	6.08	2.18	1.20	11.50	363	6.94	1.16	4.86	10.81	41	10.70	1.46	8.98	13.10	16
Rt. Ant.	12.91	3.85	1.00	23.00	189	13.36	2.14	9.00	18.00	32	16.65	1.99	13.00	20.00	10
Lt. Ant.	13.06	3.40	5.00	23.00	184	13.55	2.55	8.50	18.50	33	16.15	1.63	13.00	18.00	10
Diff. Rt.-Lt.	1.84	0.16	0	13	161	1.59	0.21	0	4	22	1.6	0.42	0	3.5	10
Thor. App.	18.80	4.60	-	23.00	212	19.63	3.29	12.00	24.00	22	18.6	3.10	15.00	24.00	10
Telson pairs	16.83	5.34	5.00	38.00	261	20.09	2.16	16.00	26.0	33	29.41	4.70	24.00	38.00	12
Rings	13.93	5.58	7.00	26.00	46	15.43	2.96	11.00	21.00	28	33.88	5.54	25.00	41.00	16

Table 6. Tukey-Kramer Multiple Comparison Results of Population differences among meristic characters. Bold indicates p-values of significance

Pop. 1	Pop. 2	Size	Rt. Ant	Lt. Ant	Thoracic legs	Telson Spine pairs	Rings
SLNY	MNJ	**<0.0001***	**0.0092 ***	**0.0286 ***	0.7959	**0.0000 ***	**<0.0001 ***
SLNY	LPMA	**<0.0001***	**0.0326 ***	0.0765	0.9261	**<0.0001 ***	**0.0000 ***
LPMA	MNJ	0.1737	0.9685	0.9663	0.8216	**0.0462 ***	**0.0097 ***

Table 7. ANOVA results comparing SLNY, LPMA and MNJ based on collection months. Individuals collected from SLNY and MNJ were both collected September and individuals collected from LPMA and MNJ were both collected in July. * indicates p-values of significance.

Meristic	SLNY ♦ MNJ	LPMA ♦ MNJ
Size	**<0.0001 ***	**<0.0001 ***
Rt. Ant.	**0.039 ***	0.6383
Lt. Ant.	0.353	0.3251
Thoracic Legs	**.0126 ***	0.9187
Telson Spine	**<0.0001 ***	**<0.0001 ***
Rings	**0.0144 ***	-

Table 8. Morphological comparisons of extant North American populations of the *Caenestheriella* and *Cyzicus* genus, adapted from Mattox 1957.

Genus	Family	Location	Carapace	Other Morphological Characteristics	Rostrum and Occipital notch
Caenestheriella	*belfragei*	TX, OK, KS	• Thick, globose shell • 21 – 35 growth lines • 7.5 mm average length • 6 mm average width/height • Prominent umbone	14 – 15 segments on second antennae	• Extended, compressed, acutely terminating rostrum in both sexes • Conspicuous and deeply cleft occipital notch
	setosa	NE, OK, KS, SD, OR, TS, MS	• Elongate • Less conspicuous umbone • ~ 15 growth lines	10 – 15 spines on telson	
	gynecia	OH, NY (?), NJ (?), MA (?)			
Cyzicus	*mexicanus*	NM, TX, AZ, KS, OK, NE, AR, IL, TN, OH, KY, PA,WV, VA, MD, Mexico, Manitoba and Alberta	• Conspicuous umbone		• Female rostrum terminates acutely • Male rostrum is broadly terminated (spatuliform) • Occipital notch is deeply cleft and terminates acutely
	morsei	ND, SD, NE, OK, IA	• Globose shell • Centrally located umbone • Many closely crowded growth lines		
	californicus	CA	• Greatly compressed shell • Small umbone which arises at the anterior end		
	elongatus	CA	• Elongate shell • ~ 7.3 mm length • ~ 4.3 mm width • Umbone not conspicuous • 18 growth lines	• Approx. 31 spines on telson • Males – two pairs of claspers as first appendages • Females – 9th and 10th appendages modified for bearing egg masses	

Table 9. Pattern of naupliar development in the conchostracan order (Schram 1986, Olesen 1999, Olesen and Grygier 2003, Pabst and Richter 2004).

Stage of naupliar development	Description
Stage 1	presence of mature biramous antennae (primary mode of locomotion); immature attenules and mandibles
Stage 2	Stage 1 with increase in naupliar size
Stage 3	mandibles mature and functional (clam shrimp now able to self feed)
Stage 4	appearance of immature maxillules and maxillae; appearance of a number of immature thoracic appendages; carapace begins to form.
Stage 5	Stage 4 with possible addition of immature thoracic appendages.
Stage 6	antennules are fully functional; thoracic appendages become differentiated; addition of immature thoracic appendages; appearance of mature telson with caudal ramus
Adult	all limbs and segments have matured (except maxillules and maxillae); thoracic appendages now used for swimming; bivalved carapace encloses entire body.

CHAPTER IV: ECOLOGICAL RELATIONSHIPS AMONG POPULATIONS OF *CAENESTHERIELLA GYNECIA* IN MASSACHUSETTS, NEW JERSEY AND NEW YORK

INTRODUCTION

To better understand urban biodiversity, it is important to study organisms of less-known habitats in addition to their well-studied counterparts. As development increases across North America, so does the number of urban, industrial and otherwise altered landscapes. Although these environments are being "redesigned" for human use there are certain species that can survive or even flourish in human-altered landscapes. This is especially true for smaller invertebrates that inhabit small or temporary pools, storm-water ponds, and artificial habitats such as puddles created and maintained by traffic of various road vehicles. The freshwater systems created as a result are composed of small habitable patches surrounded by large uninhabitable areas. The ephemeral nature and unpredictability of these habitats are the main reason for the lack of interest in long term studies necessary to acquire knowledge with regard to their communities and major ecological processes.

Vernal pool characteristics

Vernal pools are confined wetland depressions that retain water for at least two consecutive months and either dry out completely or decrease to very shallow water levels at some point annually. The temporary pools located in the northeastern United States commonly occur during the spring time and hence are given the name vernal

pools. Occasionally vernal pools become filled with autumn rains and are able to persist through the winter (Lathrop et al. 2005).

Wetland inhabitants (particularly invertebrates) are affected by the permanency of the ecosystem and their presence is determined by the duration of water in the habitat and the season of drying. There are four categories that describe the different inhabitants of vernal pools: year round residents; spring recruits, summer recruits and non-wintering migrants. Those inhabitants that are incapable of active dispersal and who survive drought periods with resistant stages are considered year-round residents. Spring recruits are those that must lay their eggs in water but can overwinter in the dry basin in various stages. Summer recruits oviposit during the dry season and overwinter as eggs or larvae. Non-wintering migrants need permanent water to survive but lay their eggs in temporary pools. They leave the pools before it dries up and return in the spring to breed. Many species who take residence for any period of time in ephemeral pools are considered generalists because they can live in both temporary and permanent aquatic ecosystems (Thorp and Covich 1991).

The outermost reaches of the vernal pool habitat is especially significant ecologically. These edges are the first to thaw in spring, providing for early access to the pools for the earliest breeding species and the outer perimeter serves as an area for the exchange of groundwater (Gamble and Mitsch 2009). They also tend to remain the warmest areas of the pool throughout the summer season. Sizes of vernal pools can range from a few yards to over an acre (NJ DEP 2005). Soils of vernal pools range in

composition from having high concentrations of clay, silicate-cemented hardpans, bedrock, volcanic mud or lava flows. These soils help to create impervious surfaces that restrict water seepage and help retain water (Wacker and Kelly 2004, Rains et al. 2008). This combination of physical characteristics creates an ecosystem that supports many endemic and endangered species of animals adapted to cyclic periods of drying and inundation.

Vernal pools serve as habitat and food sources to a variety of wildlife such as birds, amphibians and invertebrates, some of which are state-listed rare species. Invertebrates are vital to the vernal pool ecosystem, as they function as both predator and prey. Because of the shallow, inconsistent water levels and low levels of dissolved oxygen, vernal pools are unable to support fish (MacCallum 2001, Tavernini 2008, Gamble and Mitsch 2009). NatureServe compiled a list of animal species linked to isolated wetlands (vernal pools included in this category) in an effort to establish the biodiversity values of geographically isolated wetlands in the United States (2005). Clam shrimp are designated as an "At Risk" invertebrate species. This category is further subdivided into species that are critically imperiled [at very high risk of extinction due to extreme rarity (<5 populations) and very steep declines]; imperiled [at high risk of extinction due to very restricted range and very few populations (<20)]; or vulnerable [at moderate risk of extinction due to a restricted range, relatively few populations (<80) and recent and widespread declines].

Some families of clam shrimp originated in sea water but made the transition to be exclusively found in freshwater habitats (Potts and Durning 1980). Many species of *Cyzicus* are among those that partook in this habitat shift. This most likely resulted from the need to evade fast swimming predators, such as fish, and have forced them to inhabit the extreme environments that they are found today (Potts and Durning 1980).

Vernal pools formed in the Meadowlands support *Caenestheriella gynecia* populations because of the reduced predation or competition that would have been present in larger vernal pools (Schmidt and Kiviat 2007). *Caenestheriella gynecia* occurs only in the pools formed on the gas pipeline road in a 1.07 km long section and is the only known locality for this species of animal in New Jersey (Kiviat and MacDonald 2004). The objective of the third part of the project is to assess the ecological aspects of vernal pools, found in the Meadowlands, which render them habitable or inhabitable to *C. gynecia.* More specifically, I am testing the hypothesis that hydro-chemical and physical characteristics of temporary pools found in the Kane Tract area of the Meadowlands affect the presence of *Caenestheriella gynecia* within them more than climatic factors (i.e., precipitation and air temperature).

METHODS

Physical and chemical factors of individual puddles were measured to determine the reason for the presence/absence of *C. gynecia*. Eight to ten puddles were selected from a uniform transect through the study site. Clam shrimp were observed between

780 and 1,000 m of the approximately 1200 m gas pipeline road. Starting at ~ 400 m, puddles were chosen every 35 m and conspicuously marked with a black ribbon for observation between the months of May and October of 2007 and 2008.

Puddle size

The distances from the entrance of the gas pipeline tract to each puddle and the perimeter of each puddle were measured using a digital measuring wheel (DigiRoller™ Plus II). Depth of the puddle was measured using a standard metric ruler. Surface area, mean depth, and volume were determined for each pool. Surface area was calculated by measuring each pool at its longest axis and at least three cross sections perpendicular to the longest axis. Mean depth was estimated by averaging depths taken at the center of the same cross sections measured for surface area. These measurements were then used to calculate pool volume (volume = surface area × mean depth).

Water quality

Dissolved oxygen, temperature, and pH were measured using a YSI 5562 Multiprobe water meter and data were recorded one to two times a week. Nutrient/ion makeup of the puddle was determined using a LaMotte water pollution kit. Nutrient/ions tested include carbon dioxide (CO_2), silica (Si), calcium (Ca^{2+}), chloride (Cl^-). Average daily air temperature and precipitation data was retrieved from a weather recording station at the Teterboro Airport (Teterboro, NJ).

Population density

In 2008, a population density experiment was added to assess whether changes in these water quality parameters affected the presence of *C. gynecia* within the puddles. Population density was measured and standardized as the number of clam shrimp collected divided by the width of a given puddle. The number of scoops (using a sampling net) were made based on the length of the puddle, i.e., seven scoops for a 7-meter long puddle. This prevented over sampling of smaller puddles and under sampling of larger puddles by using a standard number of scoops.

Statistical analyses

One-way analysis of variance (ANOVA) was performed to test whether there were any differences among the different water quality parameters among the years and the effect, if any, on the presence of *C. gynecia*. The Tukey-Kramer multiple comparison test was performed following ANOVA to determine which (if any) months and puddles were significantly different from one another. Both tests were performed in JMP v. 8.0 statistical software. Results were deemed significant at $P < 0.05$.

RESULTS

Temperature and Precipitation

No significant difference was found in the total amount of precipitation ($P = 0.5766$) and the average temperature ($P = 0.4505$) between the two years of monitoring at the MNJ study site (Figure 28).

There was a significant negative relationship between water temperature and depth. The depth of the puddles decreased with increasing water temperature during the months of August ($P = 0.0270$), October ($P = 0.0210$) and November ($P = 0.0084$) of 2007 and May ($P = 0.0005$) and September ($P = 0.0015$) of 2008 (Figure 29). There was a significant negative relationship between air temperature and depth in September 2007 ($P = 0.0288$) and April ($P = 0.0276$) and May ($P = 0.0295$) of 2008. In July of 2007 and 2008, there was a significant positive relationship between air temperature and depth, with increasing depth with increasing air temperature ($P = 0.0292$ and $P = 0.0130$, respectively) (Figure 30).

A significant positive relationship was found between total precipitation received before sampling and the depth of the puddle, with increasing depth with increasing amount of total precipitation for both 2007 and 2008 (Figure 31). MNJ was warmer than the LPMA ($P < 0.0001$) and SNY ($P < 0.0001$) sites. There was not a significant difference in the amount of precipitation received at each of the three sampling site (Figure 32).

Water Quality

Water temperature ranged from 5.7° C – 36.4°C ($\overline{x} = 24.28$° C). There was no significant difference in water temperatures between the two years. pH ranged from very acidic, 2.6, to basic, 9.0 ($\overline{x} = 7.6$) with no significant differences between the two years. Dissolved oxygen ranged from hypoxic to supersaturated conditions (-2.0% - 214.3%, $\overline{x} = 43.45\%$; -0.60 mg/L – 15.00 mg/L, $\overline{x} = 3.27$ mg/L) with significant differences in DO between 2007 and 2008. 2008 had higher DO (% and mg/L) than

2007 ($P < 0.0001$). In 2007, some puddles experienced negative DO values with the lowest value of -2.0% saturation and -0.60 mg/L. Super-saturation was experienced in both years with percent saturation as high as 214.3% (Table 10).

Puddles salinities ranged from 0.05 – 8.54 ppm ($\bar{x} = 0.26$ ppm). There was a significant difference in salinity between the two years with 2008 possessing higher salinity values than 2007 ($P = 0.0021$). CO_2 ranged from 0.0 – 56 ppm ($\bar{x} = 10.65$ ppm). There was a slight significant difference between the two years with 2008 having higher CO_2 values than 2007. Ca^{2+} ranged from 0.0 – 664.0 ppm ($\bar{x} = 145.54$ ppm) and Cl^- ranged from 3.0 – 212.0 ppm ($\bar{x} = 58.28$ ppm). Both ion concentrations were significantly higher in 2008 than 2007 ($P < 0.0001$ and $P = 0.0010$ respectively). Si ranged from 0.0 – 10.0 ppm ($\bar{x} = 4.31$ ppm) and was significantly higher in 2007 than 2008 ($P < 0.0001$) (Figure 33).

No significant difference was found in the pH ($P = 0.6025$), DO % ($P = 0.9760$), DO mg/L ($P = 0.9992$), CO_2 ($P = 0.6354$), Ca ($P = 0.0554$) or Si ($P = 0.0556$) among puddles. There was a significant difference in water temperature ($P = 0.0001$) among puddles. Puddle 1 had the highest average temperature of 27.15 °C and puddle 7 had the lowest average temperature of 21.23 °C. There was a significant difference in salinity ($P < 0.0001$) among puddles found in MNJ. Puddle 1 had the highest salinity with 0.68 ppm and puddle 6 with the lowest salinity of 0.16. There was also a significant difference in chloride ($P < 0.0001$) among puddles. Puddle 10 had the

highest chloride concentration of 89.81 ppm and puddle 8 had the lowest chloride concentration of 38.48 (Table 11).

Water temperature in LPMA averaged 23° C, 26.4° C in SNY and 28.9° C in MNJ (Table 12). Dissolved oxygen saturation ranged from 6.8 – 26% in Pittsfield. However, the other sites had higher dissolved oxygen values – SNY at 91.9% and MNJ at 86.8%. The data presented serves as a snapshot of water quality conditions during the July sampling period in LPMA and SNY. It was used to compare monthly (July) averages of water quality conditions among puddles in MNJ.

Puddle size

Puddle depths ranged from having no water (0.00 m), during periods of evaporation, to 0.70 m after heavy rainfall events. Surface area and volume within puddles did not differ significantly between 2007 and 2008 ($P = 0.4722$ and $P = 0.9910$ respectively). However, puddles 1, 2, 3, 7, and 8 completely dried up in 2007 and puddle 6 completely dried up in 2008 (Table 13).

There was a significant differences in surface area ($P < 0.0001$) among puddles. Puddle 9 had the greatest surface area (69.60 m^2) and puddle 2 had the least (23.62 m^2). Volume differed significantly among puddles ($P < 0.0001$). Puddle 9 had the greatest volume (8.25 m^3) and puddle 2 had the least (1.96 m^3). There was a significant difference in perimeter ($P < 0.0001$) among puddles. Puddle 10 had the greatest perimeter length measuring 37.24 m. Puddle 7 had the least perimeter length measuring 17.69 m. There was a significant difference in depths ($P = 0.0002$) among

puddles. Puddle 9 was the deepest with an average maximum depth of 0.17 m. Puddle 2 was the shallowest with an average maximum depth of 0.07 m (Tables 11 and 12).

Population density

The density of *C. gynecia* did not differ significantly among puddles ($P = 0.0964$) or during different months ($P = 0.7026$) (Figure 34) (however, most clam shrimp were collected between May 13th and June 13th of 2008).

DISCUSSION

Co-occurring species

In New Jersey, *C. gynecia* shares its habitat with a variety of invertebrates and vertebrates. Some invertebrate species include pond snails (Family Lymnaeidae), mosquito larvae (Family Culicidae), dragon fly larvae and nymphs (Order Odonata), backswimmers (Family Notonectidae). Clam shrimp also share their habitat with various zooplankton assemblages which included cladocerans, ostracods, copepods, and rotifers.

In Massachusetts, there was an increase seen in the amount of amphibians present in the puddles as compared to New Jersey in addition to the previously mentioned invertebrates. These included all the metamorphic phases (eggs, tadpole, etc.) of the wood frog (*R. sylvatica*) and several salamander species (Tony Gola, MassWildlife, personal communication).

In New York, there was more diversity in the fauna that were found in the puddles. Present here were phantom midges (Family Chaoboridae), miniature freshwater clams, grey tree frog tadpoles (*Hyla versicolor)*, the red eft stage of Eastern newts (*Notophthalmus viridescens*), and cricket frogs (Genus *Acris*).

In June 2007, I found another large branchiopod cohabitating with *C. gynecia* in New Jersey. Through personal communications with Dr. Stephen Weeks of the University of Akron, we believe it to be one of three clam shrimp, *Eulimnadia texana*; the American Clam Shrimp, *Limnadia lenticularis*; or Agassiz's Clam Shrimp, *Eulimnadia agassizii*. Based on the known distributions of each, it is most likely to be either *E. agassizzii* or *L. lenticularis* as they are both found in western Masachussetts, while *E. texana* are found in southwestern America. These two clam shrimp have been known to cohabitate with each other and further morphological differentiation would be necessary to determine the true identification as the two both possess a less narrow, and more rounded carapace (translucently colored) with 7-18 growth lines (Figure 35). Passive dispersal of the cysts of *L. lenticularis* may have brought this species to NJ, the same way we speculate *C. gynecia* to have arrived at this site.

It is not uncommon for more than one species of large branchiopod to co-occur. Maedaz-Martínez et al. (1997) found eight large branchiopod species co-occurring with each other in Mexico and Arizona. These included species assemblages of the genera *Streptocephalus* (anostracan)*, Triops* (notostracan) and *Leptestheria*

(conchostracan). Waterkyn et al. (2009) found up to five coexisting species of large branchiopods in temporary wetlands sampled on the biological reserve of Tour du Valat in France. The species also included representatives of the anostracan, notostracan and conchostracan orders: *T. stagnalis, B. schaefferi, C. diaphanus, T. cancriformis* and *I. yeyetta.* Cohabitation is the likely result of the different niches that these orders occupy in the vernal pool ecosystem which decreases competition for resources. Anostracans are filter feeders, notostracans prey on other large branchiopods and conchostracans are generally benthic detritivores (Thorp and Covich 1991, Martin and Boyce 2004, Boven et al. 2008).

One vertebrate observed using *C. gynecia* habitat, at least for part of its life cycle, were snapping turtle hatchlings (Family Chelydridae). Canada geese (*Branta canadensis*) were visually observed swimming and dabbling in the puddles and raccoon (Family Procyonidae) presence can be inferred from tracks left in the mud of the puddles.

Ecological trends

Air temperature and precipitation

Precipitation is the major source of water for most vernal pools and longevity of the pools are dependent on the delicate balance among the amount of rainfall they receive during the season, evaporation and ground water exchange. Hydroperiods of vernal pools differ in the regions of the United States and are a determinant factor of zooplankton communities in temporary habitats. Although the seasonality of a region

predicts the wet and dry season of an area, there are many irregularities that can affect the length and duration of temporary pools. Such factors include differences in amount and intensity of rainfall, patterns of storms between years, evaporation and evapo-transpiration rates due to sun and wind exposure, different sediment types and the presence of macrophytes. In California, vernal pools lasted longer when precipitation was concentrated in a few month rather than equal distribution over the year. Conversely, in Massachusetts, hydroperiod maintenance was best when there was periodic precipitation (Gamble and Mitsch 2009).

Factors that affect pond duration, such as temperature and amount of precipitation, constrain the length of life and time available for reproduction. Temperature determines which species might hatch in a pool but the pool must be able to persist long enough for a species to reach maturity and produce viable eggs to maintain a population (Graham 1996). Clam shrimp that live in smaller ponds, with low average rainfall, experience a shorter total time available for development than those found in larger ponds, with higher rainfall (Marcus and Weeks 1997). The Meadowlands was warmer than the Massachusetts and New York sites, as shown in Figure 30. As there was no difference found in the amount of precipitation received at the three sites and all possess a silt-loam substrate, temperature is assumed to be the primary factor determining the presence of water at the different sites (Smith and Gola 2001, Schmidt and Kiviat 2007). Although cooler, pools in Massachusetts and New York are inundated longer, which increase the time period in which branchiopods can complete their reproductive life cycle (Brooks 2005, Pyke 2005, Gamble and Mitsch

2009). Higher temperatures combined with little rainfall does not leave enough water necessary for *C. gynecia* to complete its life cycle.

The effect of temperature on the clam shrimp habitat is further supported by the fact that 5 out of 10 puddles in the Meadowlands dried out at least once in the beginning of and the end of September/beginning of October during 2007. In 2008, 2 of the 10 puddles dried out completely at least once during the month of September. Different variables affected the evaporation of the puddles in the different years. In 2007, there was a significant negative relationship between air temperature and the depth of the pools with depth decreasing with increasing air temperature (Figure 30a). In 2008, water temperature was found to affect the depth of puddles with depth decreasing with increasing water temperature (Figure 29e). Global climate models predict an increase in maximum and minimum near-surface air temperatures. This fact coupled with an uncertainty on future patterns of frequent high intensity precipitation will eventually lead to increased summer continental drying and increased risk for drought in the coming years (Pyke 2005, Wille and Peterson 2009). An increase in air temperature or water temperature would greatly affect the habitat of *C. gynecia* and other vernal pool inhabitants as it would lead to more frequent evaporations due to changes in the balance between processes contributing to the accumulation and retention of precipitation and processes promoting the loss of water (Pyke 2005).

Water quality

Surface waters, i.e., small urban lakes and streams, wetlands, and man-made lakes, found in urban and suburban areas are vulnerable to water quality degradation. The increase in impervious cover because of development generally results in more storm water runoff and reduced ground water recharge. The loss of wetlands due to development means reduced buffering zone for heavy rainfall, thereby also contributing to floods (Amirsalari and Li 2007).

Hydrochemical characteristics influence the dynamics of zooplankton communities in temporary freshwater habitats. This is the result of fluctuations of water features due to the limited depth, water volume and surface are of these pools. Large differences of various physico-chemical features can be observed within pools located in the same geographic area (Thorp and Covich 1991, Machado et al. 1999). There is a wide variation among values for the water quality parameters within and among sampling sites (Table 13). Water chemistry data for MNJ pools reveals a range of values even though the pools are in close proximity to each other (within 35 m). Differences in water temperature among puddles can be as large as 20°C in a day. Studies have been done to find correlations between air temperature and water temperature resulting in water temperatures following air temperature with a time lag of about 2 hours with variations up to 20° C (Zedler 1987). Wille and Peterson (2009) monitored vernal pools in Oregon and found variations among temperature as much as 25° C where temperature variation was greater on clear sunny days and attenuated during overcast days.

Variations in water temperature may arise from the amount of shade provided by the surrounding canopy. Some pools were completely shaded while others were exposed to pockets of sunlight. Pools here also share the same characteristics of the LPMA in that some puddles receive direct sunlight at all times of the day and others are shaded by cottonwood, silver maple or honey locust trees. Vernal pools sampled in Massachusetts and New York are categorized as seasonal forested pools which are isolated wetlands found in temperate forests that are physically and hydrologically isolated with no connections to other surface waters. These results indicate that even though pools are proximal to each other, they can serve as micro-habitats that may result in differences in clam shrimp densities among pools. Modest changes in climate affect small pools which provide marginally suitable habitats for reproduction with the potential to shift from favorable to unfavorable conditions in short periods of time (Pyke 2005).

Water temperature can affect the presence of clam shrimp and other branchiopods in an aquatic ecosystem as most require an optimum range in temperature for their eggs to hatch and develop (Horne 1971, Thorp and Covich 1991). Mattox and Velardo (1950) studied the effect of different temperatures on the hatching and development of *C. gynecia* eggs. They determined that the minimum temperature required for the eggs to hatch was 13° C and the maximum temperature was 38° C. The mean optimum temperature for the development of these eggs was 28° C, with time to hatch increasing below 28° C and above 31° C. Belk and Belk (1975) found the optimum hatching temperature for *C. setosa* to be 25° C. During a sampling season,

C. setosa was absent during the months of April and May due to low water temperatures of 20 – 22° C which fell below the optimum hatching temperatures of *C. setosa.* Branchiopods found in West Cape, South Africa were found to hatch at their optimum temperature of 15° C where water temperatures range from 12° C and 16°C and air temperatures range between 17°C and 19° C (Day et al.2010). *Artemia* eggs can hatch with a 12°C to 25°C and Triops can hatch within a temperature range of 8° C and 22° C (Mattox and Velardo 1950). *Caenestheriella gynecia* was found to tolerate temperatures ranging from 5.7º C to 36.4º C in the New Jersey Meadowlands. This indicates that *C. gynecia* is a eurythermal species and this adaptation to a broad range of temperatures could be a key to its survival in the Meadowlands as future climatic variables will become unpredictable due to global warming.

There are a few limiting factors that contribute to the presence of invertebrates in an aquatic ecosystem. Some of the major ones include the amount of dissolved oxygen, salinity, pH and nutrient (cation/anion) makeup (Thorp and Covich 1991).

Dissolved Oxygen

Many factors influence the amount of oxygen present in an aquatic ecosystem. Temperature affects water's ability to hold oxygen as soluble oxygen decreases as temperature increases. Oxygen is more abundant in cold water than in warmer water (Table 14). The presence of submerged plants and/or algae can affect an aquatic habitat negatively and positively. As oxygen is produced by plants, it can provide an abundance of oxygen, even creating supersaturated conditions at times. However,

when these plants/algae overwhelm a system, oxygen levels decrease because of the increased oxygen demands necessary for decomposition to occur.

Both supersaturated and low oxygen levels were observed on several sampling occasions at the NJ site where thick mats of *Spyrogira* algae accumulated within a number of the puddles. Oxygen levels ranged from 1.8 to 105.2% saturation. This parameter did not have an effect on the presence of *C. gynecia* as there was no correlation found between the density of *C. gynecia* and oxygen levels ($P = 0.4758$). This is consistent with other findings demonstrating that large branchiopods, particularly conchostracans, can tolerate low levels of oxygen. Specific examples include *Cyzicus californicus,* which can tolerate 3 – 4% dissolved oxygen saturation, and *Caenestheriella setosa,* which can tolerate 1 – 2% saturation. Conchostracans have a higher tolerance for low oxygen levels and can be seen when compared to the anostracan *Branchinecta mackini*, which can tolerate oxygen concentrations of 10 – 20% (Horne 1971, Thorp and Covich 1991).

It is important to point out that these measurements were taken during the day when oxygen concentrations are high. It is possible that the range in oxygen levels would show a change if early morning and night sampling took place. Smith and Gola (2001) noticed several *C. gynecia* swimming ventral side up and gaping at the surface of the water during a night observation. They attribute this to possible low levels of oxygen during this time period.

Salinity

Freshwater invertebrates inhabit discrete sites that are typically surrounded by inhospitable terrestrial landscape (Bilton et al. 2001). Fresh water is defined as water with less than 0.5 parts per thousand dissolved salts. Salinity values are consistent with freshwater aquatic systems in the Pittsfield and Saugerties (0.03 ppt and 0.08 ppt respectively). However, salinity is significantly higher in the Meadowlands (0.28 ppt) where salinity has reached to brackish levels (0.95 ppt). This is due to the fact that the dirt road in which these pools are located is surrounded by tidal marsh. After heavy rainfall and extremely high tides, water from the marsh overflows onto the road and mixes with the water in the pools.

Salinity is known to affect the hatching rates of some branchiopods. In the notostracan *Triops cancriformus*, low salinity induces hatching (a characteristic of the initial habitat inundation) while increasing levels of salinity influences ovary maturation in the adults. As the amount of water in a habitat dries out, the salinity increases. This may be an adaptive response of *Triops* to start producing eggs in anticipation of their habitat drying out (Schönbrunner and Eder 2006). Anostracans are notorious for withstanding high levels of salinity and can hatch only in saline waters (Day et al. 2010).

pH

When Smith and Gola (2001) sampled pools at the LPMA site, they found pH values ranging from moderately to slightly acidic (6.2 – 7.6) with an average pH of 6.65.

During the 2008 sampling of the LPMA site, pH values ranged from moderately acidic to nearly neutral (5.58 – 7.25). Pools found in the New Jersey Meadowlands, on average had a pH of 6.88 although values have dipped to as low as 4.34 and as high as 9.66. This is in part due to the increased truck presence within the area. They have recently started to repair the levees on the Hackensack River and are repeatedly transporting and dumping tidal soil back and forth between puddles. Soil that is more saline has been shown to have a lower pH and puddles with high amounts of plant and animal detritus have a high pH (Al-Busaidi and Cookson 2003, Yee and Juliano 2006). Two clam shrimp, *Cyzicus grubei* and *Maghrebestheria maroccana*, inhabiting temporary pools in Portugal, have been found to tolerate slightly acidic to alkaline pH levels ranging from 6.0 to 9.3 as well (Machado et al. 1999).

CO_2 also has an affect on the pH of a freshwater ecosystem. Free CO_2 is released into the water during respiration and reacts with water to produce carbonic acid (H_2CO_3) lowering the pH (Wurts and Durborow 1992). This was observed at the MNJ site where there was a high correlation between the amount of CO_2 and the pH of the pools with increasing pH as the concentration of CO_2 increased ($r^2 = 0.02$, $P = 0.0152$).

Nutrient Makeup

Inland waters possess dilute concentrations of ions when compared to that of seawater. The primary cations found in freshwater habitats are calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^{+}), and sodium (Na^{+}). The four anions found are

bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), sulfate (SO_4^{2-}) and chloride (Cl^-). Together the cations and anions represent the total ionic salinity. The concentration of these ions can place water bodies into two categories of soft and hard water. Soft water bodies trend toward acidic waters originating from igneous rock substratum. Hard water bodies have relatively high salinities for freshwater and are usually derived from calcareous rock and are on the alkaline side (Thorp and Covich 1991).

The calcium concentrations observed at the MNJ site indicate that *C. gynecia* inhabit very hard waters (>180 ppm or mg/L). Calcium cations are important for arthropods, especially freshwater crustaceans possessing partially calcareous exoskeletons or calcareous hard shells as this is an important component in the calcification of the carapace after the molting process (Cairns and Yan 2009). Calcium is extracted from the water and is deposited as calcium carbonate in the exoskeleton. Calcium concentration is a limiting factor for survival of crustacea as animals lose most of their total body calcium during molting periods. Neonate and post-molt cycles are crucial periods of a crustacean life cycle where calcium uptake is at is greatest. When calcium concentration levels fall below suboptimal ranges, re-mineralization of the exoskeleton is compromised. Chronically low levels of calcium leads to reduced body size and delayed sexual maturity in some branchiopods. It has been found that body size is positively correlated with calcium concentrations in *Daphnia* where maturation is size dependent (Ebert 1994, Cairns and Yan 2009). Acidification of surface waters due to anthropogenic factors (air pollution, etc.) can cause the rate of shell dissolution to exceed the rate of shell deposition. Although acidic soft water could pose a

problem to some arthropods, there are some that have adapted new ways of extracting additional calcium ions by reabsorbing some from the old exoskeleton pre-molt or ingesting its recent molt (Thomas 1966, Sarac et al. 1994, Keteles and Fleeger 2001).

Chloride naturally enters an aquatic ecosystem through precipitation and geological units that contain chloride. Some anthropogenic sources include septic and industrial waste, animal waste and fertilizers. Urban aquatic ecosystems receive the majority of chloride from runoff carrying chloride from road salts used as a de-icing agent on surround impervious surfaces (Mashburn and Sughru 2004,). The EPA has identified 250 mg/L as a concentration at which chloride is acceptable in drinking and surface waters. Any values above 250 mg/L are potentially harmful to aquatic life (Amirsalari and Li 2007). The chloride concentrations at MNJ fell within the acceptable ranges for surface water and varied from as low as 0.00 mg/L to 212 mg/L.

Elevated chloride levels can reduce the diversity and impair reproduction of organisms in surface water but can also have a positive impact on an aquatic ecosystem. Aquatic animals actively uptake chloride through their gills. Both nitrate and chloride ions compete for the same site of active transport within the cells. However, nitrite is toxic to the cells and can cause dysfunctions of the oxygen-carrying pigments leading to hypoxia and death. Elevated chloride ion concentrations in the aquatic environment can help prevent the uptake of nitrate in freshwater invertebrate cells (Alonso and Camargo 2008). Although not found in this study, decreased concentrations of chloride ions may prevent certain species of zooplankton

from inhabiting or succeeding in vernal pools because of increased uptake of nitrites from the water.

Population density

Although there were significant differences in water temperature, salinity and chloride among puddles, these and the other water quality parameters tested did not have an effect on the density of *C. gynecia*. The results of this study differ from those found by Waterkyn et al. (2009). They found that local environmental factors (i.e., salinity and phosphorus concentrations) and not spatial factors (i.e., connectivity and/or isolation) were important in determining branchiopod presence in temporary pools. They found more large branchiopods in pools that were less saline and contained less phosphorus, but were more turbid and had a greater number of zooplankton taxa found within. They also saw that different branchiopods appeared during different inundation periods with the most appearing after the first inundation (out of a total of three inundations). They attribute this to ample rain and high temperatures during the first inundation. The population density experiment of this study was similar to that of Waterkyn et al (2009) in that the majority of *C. gynecia* in this study were collected during the one month following the first inundation of the pools in the Meadowlands and steadily declined thereafter.

Tavernini (2008) found differences in zooplankton densities during their two year study of temporary pools in Italy. In 2001, there was an explosive growth of zooplankton following the first inundation of the season followed by a decline during

mid-June to mid-July, when there was lower precipitation. Another peak was seen after storm events refilled pools at the end of July. Conversely, in 2002, some of the pools maintained their volume throughout the season and only experienced one peak in the abundance of zooplankton.

The results of this study presented a wide range in the hydro-chemical and physical characteristics of the ephemeral pools in which *C. gynecia* seem to tolerate. My hypothesis was rejected as neither these characteristics nor climatic variables affected the presence of the clam shrimp in New Jersey during the two sampling seasons. It can be inferred that *C. gynecia* has become well adapted to the phenology of their wetlands and their life-history traits and hatching patterns have adjusted accordingly as the hatching of branchiopod dormant eggs is normally triggered by a combination of environmental characteristics (Waterykyn et al. 2009).

Additional sampling activities are necessary to determine inter-annual patterns in *C. gynecia* densities and dynamics to see if they are repeated every year. Additionally, comparative population density and hydro-chemical studies among the different populations (New York, New Jersey and Massachusetts) of *C. gynecia* within a growing season (April – October) could reveal the effect of these variables in the future.

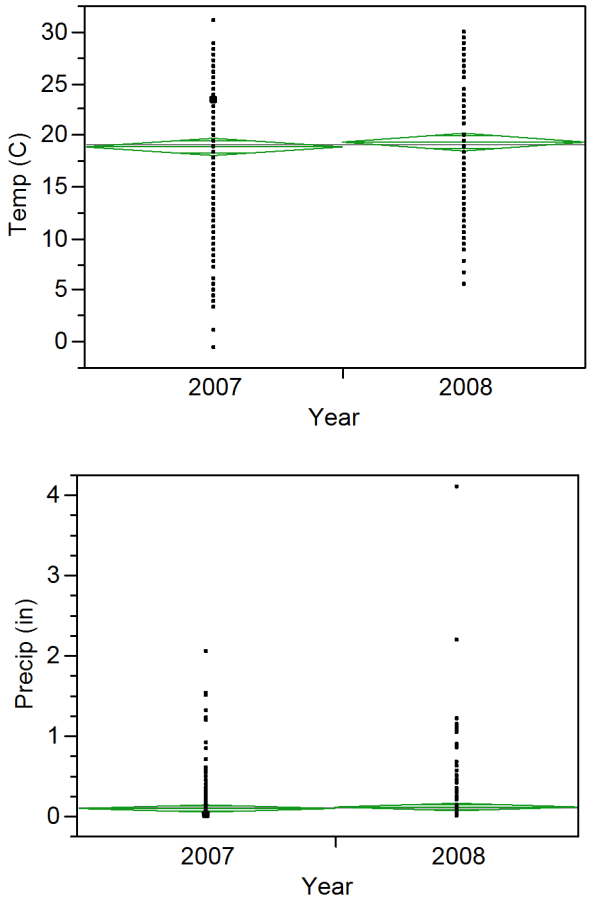


Figure 28. ANOVA results comparing the temperature and amount of precipitation received at the MNJ study site during the 2007 – 2008.

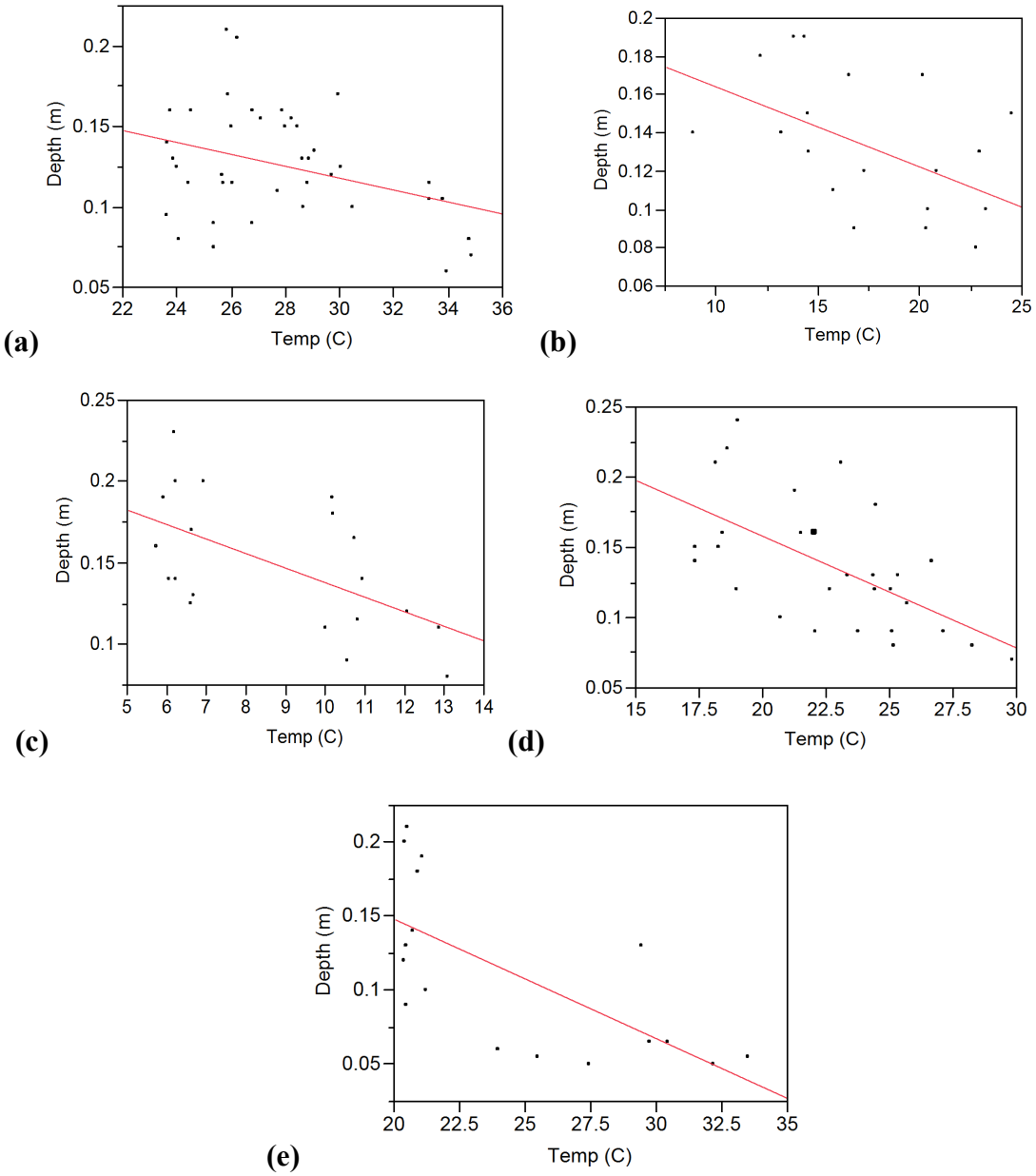


Figure 29. ANOVA results showing negative relationships between water temperature and depth of puddles in New Jersey (a) August 2007 (b) October 2007 (c) November 2007 (d) May 2008 (e) September 2008.

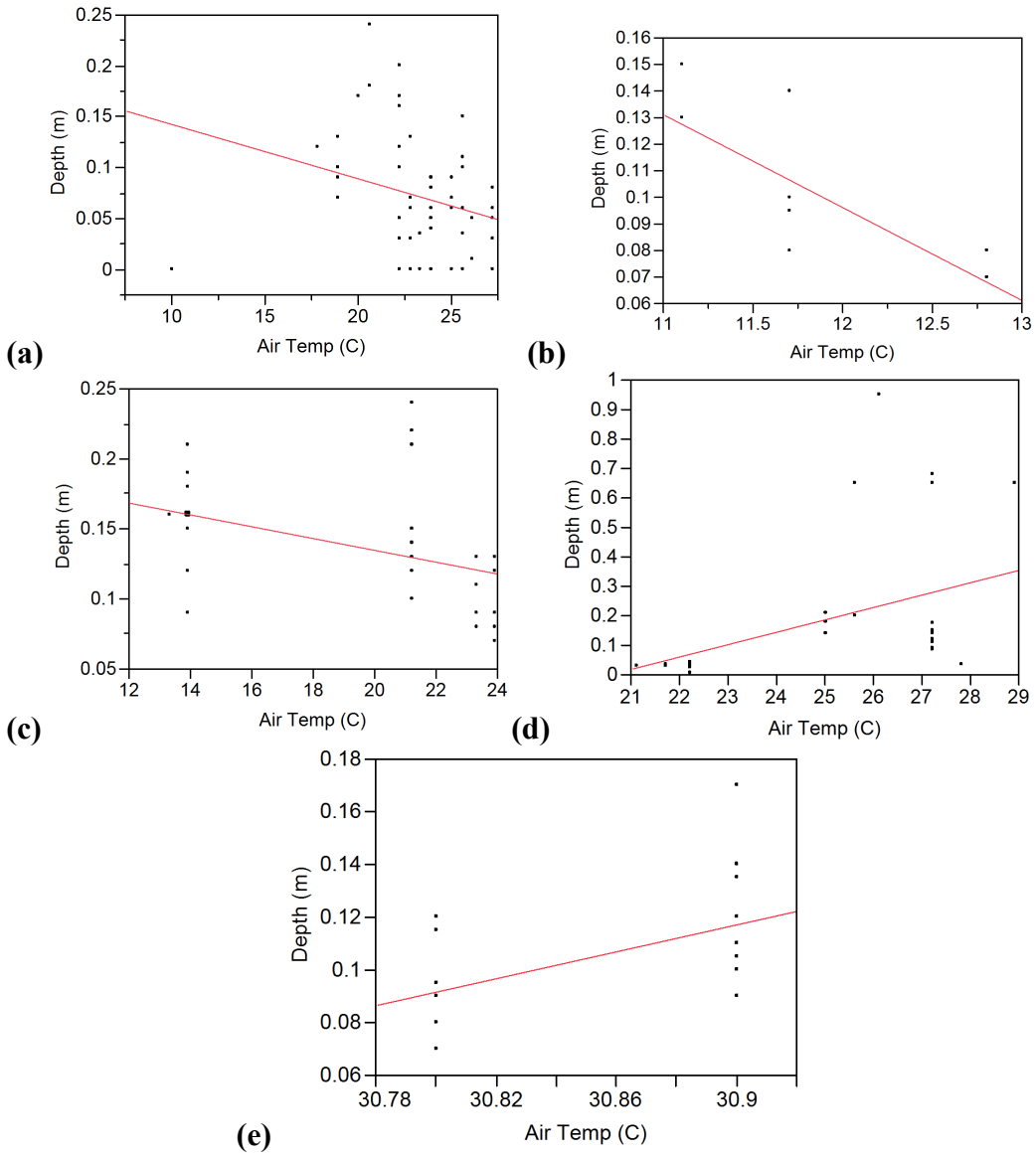


Figure 30. ANOVA results showing relationships between air temperature and depth of puddles in New Jersey (a) September 2007 (b) April 2008 (c) May 2008 (d) July 2007 (e) July 2008.

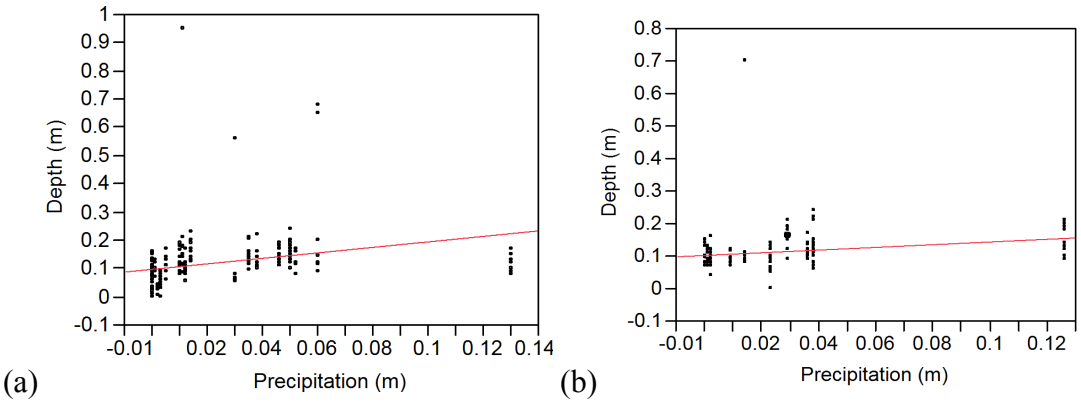


Figure 31. ANOVA results showing positive relationships between amount of precipitation received between sampling dates and the depth of the puddles (a) 2007 (b) 2008.

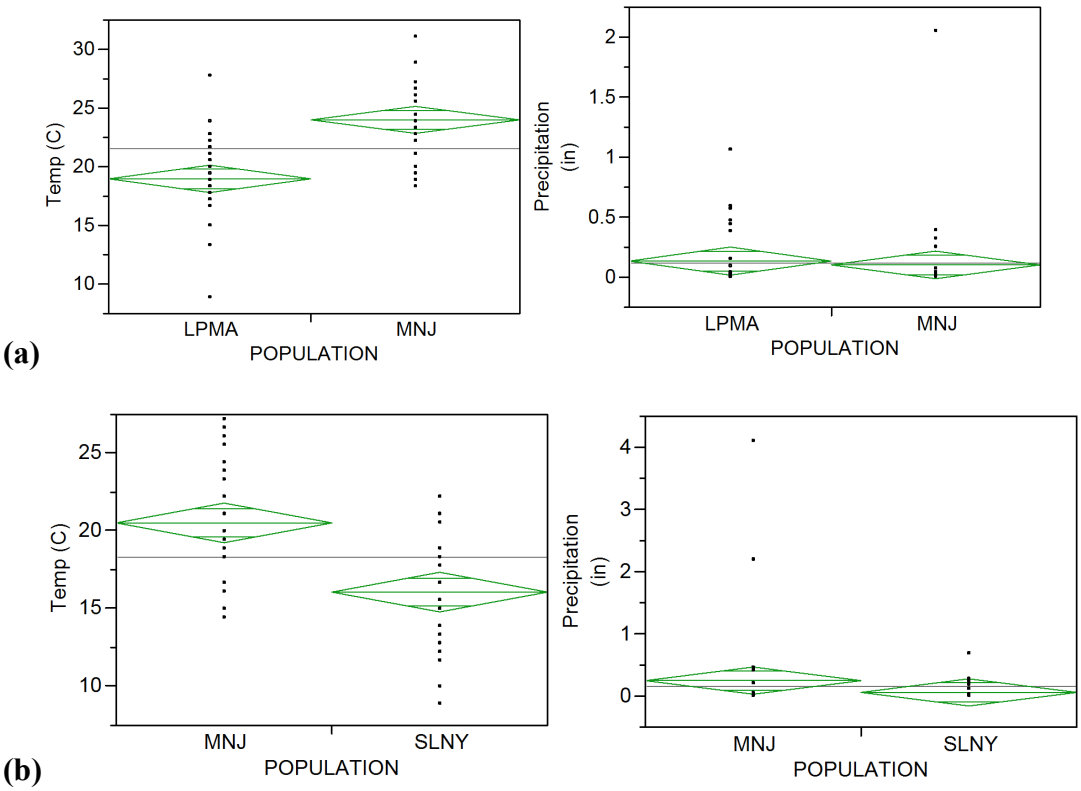


Figure 32. ANOVA results displaying significant differences in temperature among the three study sites. There was no significant difference in precipitation. Temperature and Precipitation were compared during the same months of collection. (a) July 2007 (b) September 2008. LPMA: Massachusetts site. MNJ: New Jersey site. SLNY: New York site.

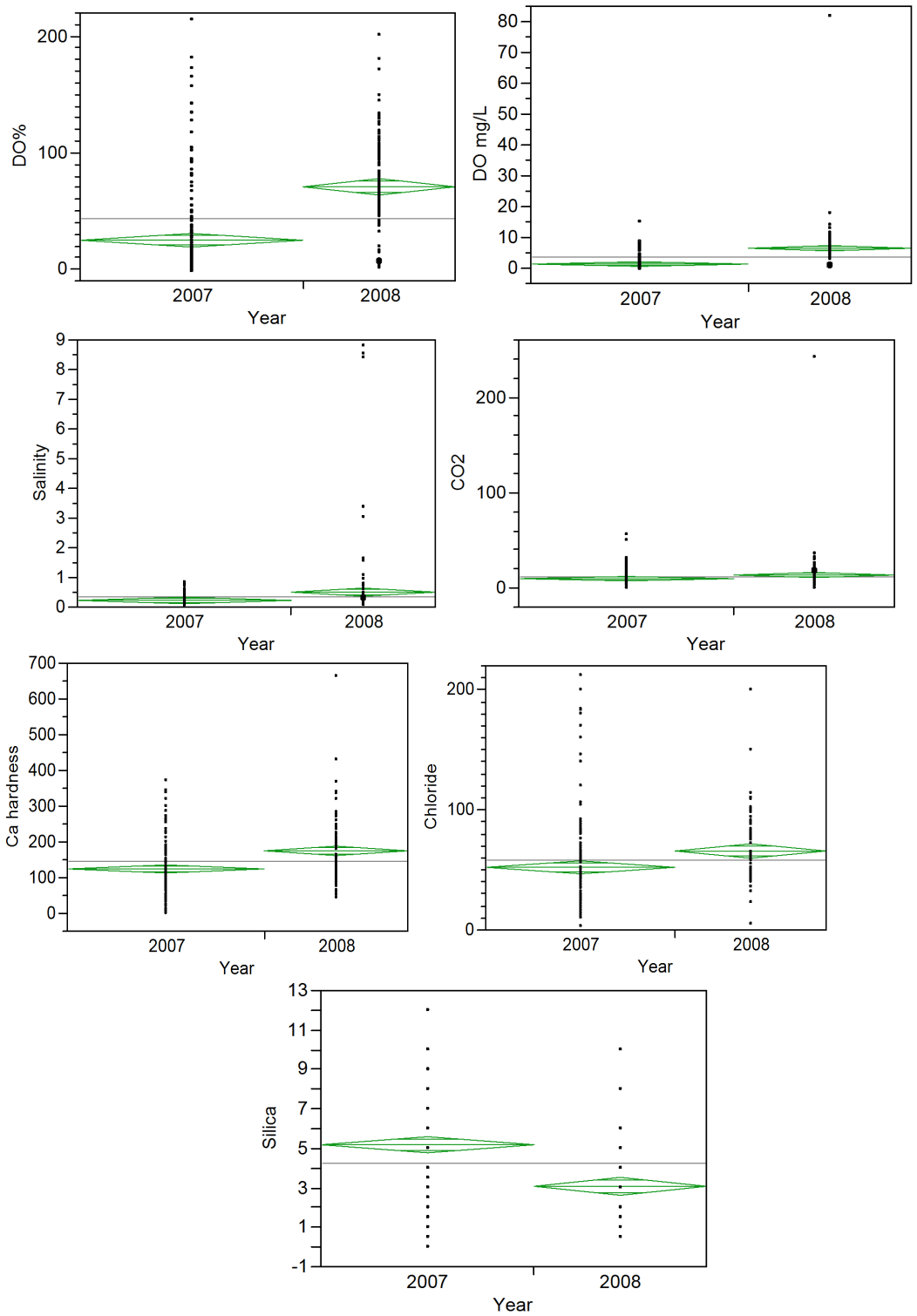


Figure 33. ANOVA results comparing water quality parameters between the two years of the study.

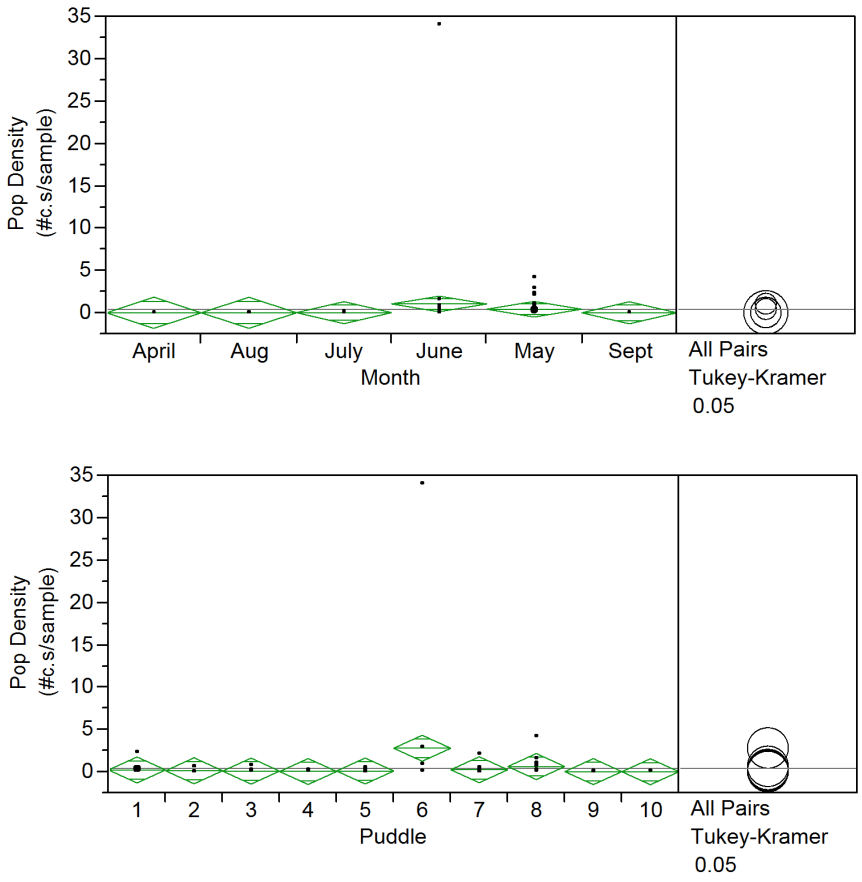


Figure 34. Population density relationships between months and among puddles for NJ.

Figure 35. Unidentified clam shrimp co-occurring with *C. gynecia* in 2007.

Table 10. Water chemistry of pools containing *Caenestheriella gynecia* as the average of three sites in Pittsfield, MA, one site in Saugerties, NY and the main study site in the New Jersey Meadowlands. New Jersey values are the average of the 10 puddles at this site. All measurements were taken around midday (12:00 – 1:00 PM).

Location	Temperature °C	DO %	DO mg/L	Salinity ppt	pH
LPMA	22.91	16.77	1.38	0.02	6.26
SNY	26.36	91.9	7.4	0.08	6.99
MNJ	28.91	86.8	6.85	0.28	6.88

Table 11. Rank of puddles (highest to lowest) of water quality parameters and puddle size dimensions of significance.

Temperature	Salinity	Chloride	Surface Area	Volume	Depth	Perimeter
1	1	10	9	9	9	10
2	2	2	10	10	1	9
4	3	3	4	4	5	4
5	5	1	5	5	10	5
10	7	5	3	1	6	3
6	9	4	1	3	7	1
9	10	9	8	7	4	8
3	4	7	6	8	8	6
8	8	6	7	6	3	2
7	6	8	2	2	2	7

Table 12 Surface area and volume for the 10 puddle sites of New Jersey.

Puddle #	Coordinates	Year	Surface Area (m^2)	Volume (m^3)
1	N 40.82747	2007	0.00-64.76	0.00-20.011
	W 74.04084	2008	11.91-94.53	0.00-12.28
2	N 40.82715	2007	0.00-50.53	0.00-10.05
	W 74.04072	2008	0.00-55.57	0.00-6.30
3	N 40.82679	2007	0.00-76.20	0.00-5.16
	W 74.04062	2008	20.82-77.52	0.76-17.31
4	N 40.82656	2007	0.05-175.06	0.01-14.55
	W 74.04049	2008	16.10-115.57	0.99-15.03
5	N 40.82586	2007	0.76-77.52	0.01-8.15
	W 74.04036	2008	36.79-99.79	1.96-14.97
6	N 40.82526	2007	0.27-53.61	0.01-4.94
	W 74.04020	2008	0.00-36.57	0.00-3.14
7	N 40.82496	2007	0.00-60.78	0.00-17.74
	W 74.04009	2008	1.74-47.47	0.09-4.35
8	N 40.82473	2007	0.00-63.83	0.00-6.14
	W 74.03997	2008	3.23-61.57	0.14-6.68
9	N 40.82449	2007	0.21-116.81	0.03-32.67
	W 74.03988	2008	33.15-144.14	3.15-20.66
10	N 40.82422	2007	0.25-115.78	0.03-16.44
	W 74.03985	2008	8.06-125.40	2.27-15.47

Table 13. Range of Physico-chemical characteristics of the 10 pools during the 2007-2008 research period (April – November). Puddle (P), Perimeter (Per), Depth (D), Water Temperature (T), Dissolved Oxygen (DO), Salinity (Sal), Carbon Dioxide ($CO_{2)}$, Calcium (Ca), Chloride (Cl), Silica (Si)

P	Year	Per (m)	D (m)	T (°C)	pH	DO %	DO (mg/L)	Sal (ppm)	CO2 (ppm)	Ca (ppm)	Cl (ppm)	Si (ppm)
1	2007	0.0 - 46.1	0.00 - 0.65	6.6 – 36.0	5.5 - 8.7	-2.0 - 181.5	0.02 - 15.0	0.15 - 0.83	0.0 - 25.0	47.0 - 344.0	10.0 - 84.0	2.0 - 10.0
	2008	4.3 - 45.2	0.05 - 0.24	18.2 - 34.1	4.4 - 8.7	1.0 - 180.4	0.09 - 12.91	0.15 - 3.37	0.0 - 32.0	64.0 - 664.0	52.0 - 150.0	1.0 - 8.0
2	2007	0.0 - 41.6	0.00 - 0.13	6.9 - 36.4	5.5 - 8.3	0.5 -142.1	0.05 - 6.90	0.14 - 0.71	0.0 - 56.0	60.0 - 272.0	35.0 - 180.0	2.0 - 10.0
	2008	0.0 - 40.2	0.05 - 0.24	12.7 - 34.5	4.7 - 8.7	0.8 - 118.1	0.07 - 81.8	0.20 - 8.40	0.0 - 26.0	100.0 - 320.0	44.0 -100.0	0.5 - 8.0
3	2007	3.0 - 32.6	0.01 - 0.15	6.7 - 29.9	5.5 - 8.6	-1.4 - 165.0	-0.40 - 3.99	0.10 - 0.52	0.0 - 24.0	40.0 - 372.0	28.0 - 140.0	3.0 - 10.0
	2008	19.0 -35.7	0.00 - 0.18	19.5 - 28.1	4.8 - 9.0	1.0 - 201.1	0.09 - 17.76	0.16 - 8.80	0.0 - 36.0	60.0 - 368.0	48.0 - 200.0	0.5 - 5.0
4	2007	1.2 - 40.4	0.03 - 0.16	6.6 - 31.7	5.5 - 8.3	-0.2 - 214.3	-0.60 - 6.93	0.05 - 0.36	0.0 - 50.0	32.0 - 320.0	26.0 - 72.0	1.0 - 10.0
	2008	2.9 - 50.0	0.05 - 0.16	19.4 - 35.0	6.4 - 9.7	0.9 -133.4	0.08 - 9.49	0.11- 8.54	0.0 - 23.0	44.0 - 276.0	42.0 - 110.0	0.5 - 8.0
5	2007	5.4 -39.4	0.03 - 0.20	5.9 - 33.1	7.1 - 8.5	-0.5 - 172.4	-0.04 - 7.61	0.07 - 0.55	0.0 - 50.0	32.0 - 338.0	22.0 -104.0	2.0 - 10.0
	2008	9.4 - 47.2	0.07 - 0.21	17.9- 34.3	6.1 - 9.1	1.0 - 131.2	0.09 - 11.30	0.13 - 0.39	0.0 - 24.0	44.0 - 320.0	36.0 - 98.0	0.5 - 3.0
6	2007	2.9 - 35.0	0.01 - 0.65	5.7 - 30.4	7.0 - 8.2	-0.6 -156.9	-0.05 - 8.51	0.05 - 0.30	0.0 - 26.0	0.0 - 200.0	18.0 - 88.0	0.5 - 12.0
	2008	0.0 - 28.1	0.00 - 0.70	17.4- 35.1	4.3 - 8.8	1.0 - 126.3	0.09 - 9.83	0.08 - 0.39	0.0 - 25.0	64.0 - 276.0	32.0 -100.0	0.5 - 5.0
7	2007	2.9 - 39.9	0.01 - 0.65	6.0 - 26.9	7.0 -8.0	-0.3 - 127.5	-0.03 - 7.50	0.08 - 0.43	6.0 - 24.0	0.0 - 236.0	12.0 - 60.0	0.0 - 10.0
	2008	5.9 - 37.8	0.05 - 0.15	14.9 - 25.5	4.7 - 8.7	1.1 -116.0	0.11 - 10.59	0.10 - 0.48	0.0 - 20.0	80.0 - 340.0	40.0 - 91.0	1.0 - 8.0
8	2007	0.0 - 28.9	0.00 - 0.22	6.2 - 28.7	7.1 - 8.2	-0.5 - 134.2	-0.04 - 8.02	0.06 - 0.24	4.0 - 27.0	24.0 - 252.0	3.0 - 70.0	2.0 - 10.0
	2008	6.9 - 33.1	0.06 - 0.15	13.4 - 26.3	5.2 - 8.9	1.1 - 111.7	0.10 - 9.77	0.10 - 0.71	0.0 - 24.0	52.0 - 248.0	5.0 - 80.0	1.0 - 10.0
9	2007	3.6 - 43.1	0.01 - 0.65	6.2 - 30.9	7.1 - 8.1	-0.6 - 141.9	0.00 - 5.90	0.08 - 0.38	0.0 - 20.0	48.0 - 222.0	12.0 - 82.0	2.0 - 6.0
	2008	29.9 - 46.1	0.11 -0.12	16.3 - 29.4	5.6 - 9.0	1.2 - 99.2	0.11 - 9.28	0.13 - 0.37	0.0 - 32.0	88.0 - 430.0	40.0 - 110.0	0.5 - 5.0
10	2007	2.7 - 48.4	0.02 - 0.21	6.2 - 32.5	7.2 - 8.2	-2. - 117.1	-0.16 - 6.60	0.06 – 0.40	0.0 - 17.0	12.0 - 212.0	12.0 - 212.0	0.0 - 10.0
	2008	15.9-56.1	0.70 - 0.19	15.9 – 34.0	2.6 - 8.8	1.1 - 149.3	0.11 - 11.30	0.07 - 0.31	0.0 - 30.1	90.0 - 210.0	40.0 - 102.0	1.0 - 8.0

Table 14. Oxygen saturation in water at various temperatures.

Temperature	5 °C	15 °C	25 °C	35 °C
mg O_2 / liter	12.770	10.084	8.263	6.949

CHAPTER V: GENETIC RELATIONSHIPS AMONG POPULATIONS OF *CAENESTHERIELLA GYNECIA* IN MASSACHUSETTS, NEW JERSEY AND NEW YORK

INTRODUCTION

Caenestheriella gynecia belongs to one of three genera of clam shrimp found to inhabit temporary freshwater habitats in North America. *Caenestheriella* consists of three species: *Caenestheriella setosa, C. belfragei,* and *C. gynecia*. Of these three species, *C. gynecia* is the only parthenogenetic one with no males present and is also one of only three species of conchostracans in the world for which males are "not yet known." (Mattox 1950, Emberton 1980, Lively and Johnson 1994).

Many vernal pool inhabitants reproduce by parthenogenesis and exhibit clonal behavior. Obligate parthenogens produce clones as offspring whose genetic makeup is identical to theirs (no genetic recombination occurring). Females produce daughters asexually which double in number with each consecutive generation. This form of reproduction enables successful colonization of new environments as only one female is necessary to found a new population (Herbert 1991, Kearney 2005, Vorburger 2006, Schön 2007). Clonal reproduction can be found among most major groups of eukaryotic species, except for birds and mammals (Halkett et al. 2005, Charlesworth 2006).

In addition to obligate parthenogens, there are some species of animals that are facultative or cyclical parthenogens. Facultative parthenogens are females that can reproduce sexually (when males are present and available) but exclusively produce

female offspring asexually under conditions where sexual reproduction is prohibited (i.e., unfavorable or deteriorating habitat conditions) (Matsuura et al. 2004, Corley and Moore 1999). Cyclical parthenogens alternate between asexual and sexual reproduction where there is only one sexual generation per year followed by *n* parthenogenetic generations (Rispe and Pierre 1998). Some cyclical organisms include aphids, *Daphnia* and rotifers. Besides the benefit of producing genetically diverse offspring, there appears to be an ecological benefit to cyclical parthenogenesis. Ecologically, parthenogens are more common in freshwater than marine habitats, more common in high latitudes and altitudes, and are more commonly found in temporary habitats. The sexual eggs produced by aphids and *Daphnia* are those that are cold and drought resistant and are able to survive in these marginal habitats (Lively and Johnson 1994, Rispe and Pierre 1998, Simon et al. 2002, Berg 2005).

Some parthenogenetic aquatic invertebrates produce diapausing cysts. These resting eggs are collectively found in the upper centimeters of soil which are known as cyst banks. Cyst banks allow the populations of these organisms to persist even in extreme environmental conditions and can serve as a reservoir of alleles necessary for the maintenance of genetic variation for future populations (Boileau and Herbert 1991, Berg 2005, Muñoz 2010, McCafferty et al. 2010).

To better understand the ecology and evolution of temporary freshwater inhabitants, one necessary first step is to understand the extent of gene flow among populations (McCafferty et al. 2010). Genetic tools can provide invaluable information to

understanding the ecological and evolutionary processes that shape genetic differentiation in diapausing aquatic invertebrates (Muñoz 2010). Before genetic profiling, scientists used sexual dimorphism as evidence of clonality. This was problematic as some species once considered clonal may have males that are rare, may be too morphologically similar to be noticed as such, or may require environmental conditions different than those of a study site to manifest (Figueroa et al. 1999, Halkett et al. 2005). In this study I observed natural clonal distributions across a 2-yr interval by employing two molecular techniques, RAPD analysis and mtDNA sequencing. As a strictly parthenogenetic species, populations of *C. gynecia* should have little to no genetic variation when compared to each other and among pools found in the Meadowlands.

Specific questions raised were as follows:

1. ***What is the population structure of C. gynecia? Are there different clones present in a given population of C. gynecia?*** Vernal pools lack ongoing connectivity with other water bodies, essentially making them isolated islands surrounded by inhospitable habitat. Although *C. gynecia* is a parthenogenetic species, different clones may appear due to limited genetic reorganization (i.e., chromosomal modification, gene conversion) which has been documented in other clonal organisms (Halkett et al. 2005). If more than one clone is present per puddle, mtDNA sequencing can determine if clones may have been formed from the accumulation of mutations at a single locus point. Individuals from NJ, NY, and MA populations of *C. gynecia* were analyzed to determine clonal diversity among populations.

2. ***In subpopulations of C. gynecia, how many of each clone is present?*** If multiple clones are shared among the NJ study site, one can postulate that dispersal of clones may be occurring through different forms of passive dispersal events (i.e., flooding, wind blown cysts). These dispersal events would help to supply these populations with individuals possessing "new" genetic information (Holland and Jenkins 1998). Alternatively, multiple clones may result from multiple founder events due to the presence of cyst banks (Boileau and Hebert 1991).

3. ***Are there any shared clones among populations of C. gynecia?*** It has been shown that some obligate parthenogenetic aquatic invertebrates exhibit clonal behavior and have identical genotypes that are shared among highly distant wetlands (Muñoz 2010). If there are any shared clones among the NJ, NY and MA populations, one can postulate that dispersal of clones may be occurring by active dispersal events (i.e., waterfowl mediated dispersal, ATVs). Nearly all populations of *C. gynecia* have been discovered in tracks filled with water made by vehicles, from the wheels of wagons from the past to the ATV vehicles of the present (Schmidt and Kiviat 2007, Smith and Gola 2001, Mattox and Velardo 1950). Smith and Gola (2001) noted that some populations are also found along or near railroad grades. In addition, direction of dispersal may be determined as populations in close proximity to the site of origin would be expected to possess higher genetic diversity than those that were derived from them. This outcome would result from the presence of the original colonists' genetic diversity plus any new mutations that had accumulated in their descendants (Boileau and Hebert 1991). If populations of *C. gynecia*

originated from Ohio, western populations (New York and New Jersey) would have higher diversity than eastern populations (Massachusetts).

METHODS

In order to understand the pattern and maintenance of genetic variation, if any, within *C. gynecia*, genetic variation and structure both within and among pools over the species range was analyzed.

Populations examined for genetic variability included those found in the Meadowlands, NJ; populations found in the Hudson Valley – Saugerties, NY and Hyde Park, NY; and in Pittsfield and Lenox, Massachusetts. Population-level relationships were estimated by combining two sensitive molecular approaches: random amplified polymorphic DNA (RAPD) analysis and mitochondrial DNA (mtDNA) sequencing.

RAPD analysis was chosen as an initial technique because of its sensitivity in detecting genetic diversity among populations of species with little to no genetic information available about them. RAPD is a polymerase chain reaction based method that can be performed without any previous knowledge of specific DNA sequences of the species under study (Williams et al. 1990, Lynch and Milligan 1994, Micheli et al. 1994, Martinez et al. 2006, Huang et al. 2009) (Figures 36 and 37). It uses arbitrary primers to detect changes in the DNA sequence at sites in the genome which anneal to the primer. The DNA segments are inherited in a Mendelian fashion and can be used to construct

genetic maps in a variety of species. Polymorphism is detected as differences between the patterns of individuals' DNA fragments amplified from the different DNAs using a given primer(s) (Williams et al. 1990, Bagley et al. 2001) (Figure 38).

RAPD has been successfully used to study genetic structure in plants, birds, snails, and invertebrates (DeWolf et al. 2004, Yoke-Kqueen and Radu 2006, Martinez et al. 2006, Micheli et al.1994, Li and Jin 2006). Other uses include genetic mapping, population and pedigree analysis, phylogenetic studies, bacterial strain identification, evaluation of anthropogenic stress in aquatic ecosystems and assessing the impacts of known stressors on genetic diversity (Micheli et al. 1994, Pascual et al. 2006, Bagley et al. 2001).

Advantages of using RAPD techniques include:

1. **Time and Expense**. RAPD is an inexpensive and expedient approach to measuring genetic diversity. In addition, it requires only small quantities of DNA – advantageous for clonal organisms as most are tiny (Bagley et al. 2001, Sun and Wong 2001, Halkett 2005).
2. **Use of standard materials**. RAPD fragments can be separated by size using a standard agarose gel which is visualized by staining the gel with ethidium bromide. This eliminates the need for radio-labeled probes (Lynch and Mulligan 1994, Pascual et al. 2006).
3. **Multiple polymorphic fragments**. Intermediately sized primers (>10 base pairs) produce multiple amplifiable, polymorphic fragments for each set of primers in each genome (Lynch and Mulligan 1994).

4. **No DNA discrimination**. RAPD primers do not discriminate between coding and non-coding regions because they consist of random sequences (although it most often contains only non-coding DNA). This allows for more random sampling of a species genome compared to conventional (i.e., microsatellite sequencing) methods (Lynch and Mulligan 1994).
5. **Sensitivity**. RAPD can be more sensitive than allozymes in detecting genetic structuring between populations with increasing distributional range (De Wolf et al. 1998, Sun and Wong 2001).
6. **Availability.** Obtaining more than a dozen microsatellite panels to assess polymorphisms is highly unlikely for less studied species. The microsatellite markers that do exist are for commercially or scientifically important species. To develop and produce new microsatellites would require a greater amount of time and expense (Bagley et al. 2001).

There are also some disadvantages of the RAPD technique that serve as a cause for many investigators to be discouraged from using it. These include:

1. **Low reproducibility**. The ability for RAPD techniques to produce reproducible bands is affected by a number of reasons: variations in reaction mixtures such as the concentration of primers or template material (Burnett 2003); type of DNA polymerase used; and temperature profile characteristics of the PCR thermocycler used (Pascual et al. 2006). In addition, differential DNA preparation techniques can affect how primers anneal to DNA templates (i.e., ethanol precipitated DNA vs. DNA wound on a glass rod) (Micheli et al. 1994).

2. **Amplification of dominant markers**. RAPD only amplifies dominant markers but cannot specify between homozygous and heterozygous individuals. Alternative alleles fail to produce a fragment so that no band is developed and is recorded as "null/0." This often leads to inaccurate gene frequency estimates for those loci used to measure gene diversity within populations, usually leading to inflation of the estimates when small population sample sizes are used. Gene diversities can also be inflated due to the fact that RAPD profiles are generated from the total genomic DNA and are not able to discriminate between nuclear DNA and the mitochondrial DNA. (Lynch and Milligan 1994, Sun and Wong 2001, Zarattini 2002, Burnett 2003).
3. **Co-migration**. There is the possibility that co-migration of different loci with similar molecular weights may lead to indistinguishable band patterns on a standard gel. Using a polyacrylamide gel usually corrects this problem as it is more sensitive than a standard agarose gel (Lynch and Mulligan 1994).
4. **No preliminary information.** Because there is usually no preliminary information available on the pedigree of a species to compare to, assigning markers to a specific locus can be met with uncertainty (Lynch and Mulligan 1994).

Mitochondrial DNA sequence variation is a powerful genetic marker for population genetic analyses and has been used extensively in the past three decades because of its high level of genetic variation. The control region of mtDNA is especially variable and is responsible for the replication and transcription of this organelle (Kuhn et al. 2008).

Animal mtDNA is composed of a circular double-stranded molecule with a typical 14 – 16 kb length range. It consists of a coding and a non-coding region (also referred to as the control region). 12S rRNA, *large subunit* 16S rRNA (16S) and *cytochrome c oxidase subunit 1* (COI) are some of the commonly used sequences to detect variation within recently diverged lineages of arthropods <5 mya (Wetzer 2001). To date, mitochondrial DNA genes used to elaborate relationships among the branchiopod classes are 12S rRNA, EF1α (Braband et al. 2002), COI, 16S, 28S rRNA, *small subunit* 18S rRNA (18S) (deWaard et al. 2006) and ITS1 (Baxevanis et al 2006).

All published work on *C. gynecia* (a total of five papers) has only reported on occurrences and general habitat characteristics of the organism (Smith and Gola 2001). Researching the genetics of *C. gynecia* populations will give insight towards the study of genetic structure within parthenogenetic species with the use of the genetic markers *large subunit*16S rRNA (16S) and *small subunit* 18S rRNA (18S). These markers have proven to be informative within other parthenogenetic species, such as *Artemia* (deWaard et al. 2006). 16S rRNA is the small subunit of the ribosome in prokaryotes. The gene that codes for 16S rRNA is ~1550bp containing highly conserved regions interspersed with variable regions. Conserved regions of the gene can be used to design universal primers for the amplification of a wide range of microorganisms and variable regions can be used to distinguish various species from one another (Amann et al. 2000). 18S rRNA is the eukaryotic equivalent of 16S and is ~1900bp (Kimball 2010). In addition, mtDNA sequencing has been used to study genetic variation among geographically separated populations for over 25 years now (McCafferty et al. 2010).

As RAPD would only be able to reveal the presence of different clones based on absence/presence of bands in banding patterns, sequencing of mtDNA would tell specifically where the difference occurs in the DNA sequence, estimate the evolutionary relationship among the mtDNA haplotypes and also estimate the frequency and occurrence of haplotypes within and among populations. Clonal reproduction prevents the reshuffling of alleles among and across loci and affects the dynamics of genes at all levels (Halkett et al. 2005).

DNA extraction

Individuals were dissected and their digestive tracts removed so that the DNA of their gut contents would not contaminate the sample (Mattox and Velardo 1950). Genomic DNA was extracted from the remaining animal section by placing it in 250 µl aliquots of proteinase K extraction buffer (2M Tris. HCl, pH 8.0; 0.5M EDTA, pH 8; 2.5 M NaCl; 10% SDS; 10 µl proteinase K; stored at 4ºC) and was incubated at 55ºC overnight. DNA was extracted with 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1); shaken 10 min and followed with a 5-min high-speed microcentrifuge (10,000 rpm) spin at room temperature. This procedure was performed twice more. Ethanol precipitation of DNA was carried out by adding equal volume of sodium acetate to supernatant and 800 µl of 100% ethanol, followed with 10-min high speed centrifuge. The DNA pellets were washed with 400 µl of 70% ethanol, dried by a high-speed spin for 20 min in a SpeedVac Concentrator (Savant Instruments, Inc.; Farmingdale, New York, U.S.A.), resuspended in 100 µl sterile water, incubated for 30 min at 37º C and stored at 4ºC. Estimation of DNA concentration was made by electrophoresis and ethidium- bromide staining of 4 µl of

DNA on a 0.8% high-melting agarose/1X TAE (Tris-acetate EDTA) gel at room temperature and 80 volts.

RAPD analysis

RAPD reactions were performed in a final volume of 25 µl, containing 25 pmol RAPD primer (Table 15), 50 – 100 ng template DNA and a standard quantity of Ready To Go RAPD Analysis mixture (Amersham Pharmacia Biotech, Inc. #27-9502-01). The mixture was denatured for 5 min at 95°C followed by 45 cycles of 1 min denaturation at 95°C, 1 min annealing at 36°C and 2 min extension at 72°C. The amplified product was resolved by electrophoresis on 1.5% agarose gel in 1x TAE buffer for 1 hour at 100 volts. The gel was stained with ethidium bromide and immediately photographed under UV light. Sizes were inferred by comparison with a 100 bp ladder. Six primers (P1 – P6) obtained from the Ready to Go RAPD analysis kit was tested.

Six oligonucleotide (10 bp in length) RAPD primers, provided by the Ready-To-Go RAPD Analysis Kit (Amersham Pharmacia Biotech, Inc. #27-9502-01) were tested for selection in this experiment (Table 15). In a first step of the primer selection, DNA of two individuals from NJ and one from MA were amplified with each primer. Each PCR experiment was repeated in a second PCR-run with new template DNA. In a second step, the RAPD primers which revealed reliable and reproducible band patterns in both PCR-runs and produced at least six polymorphic bands were selected. Finally, two RAPD

primers (Primers 1 and 2) were retained for the assessment of the genetic structure of the analyzed *C. gynecia* populations.

RAPD bands produced were scored as present/absent (1 / 0) and entered into a binary data matrix. A conservative approach was taken when scoring bands as absent and present. All possible bands were attempted to be identified, including many that probably would not normally be scored for various reasons. One such reason is that differential band strengths can be due to the quality and quantity of the DNA used. High quality DNA will produce bands of greater intensity as would a high amount of DNA. Due to the size of *C. gynecia*, faint bands were considered to be the result of low amounts of DNA available. Each individual was genetically characterized by the presence or absence of each amplification product. Selection of primers was based on reproducibility of the RAPD profiles and its consistency of producing polymorphic bands for DNA concentration (1 – 5 ng μl^{-1}) (Williams et al. 1990).

mt DNA sequencing

16S and 18S regions of mtDNA were examined in *C. gynecia* using the primers developed by deWaard et al. (2006), Carvalho et al. (2004), and Duff et al. (2004) (Table 16).

PCR conditions

Each 21 µl PCR reaction contained 1µl of DNA template, 2 µl of 10x PCR buffer, 2 µl each of the forward and reverse primer for each sequence at 5µM (0.5 µM final), 2 µl of dNTP, 11 µl of dH_2O and 1 µl of *taq* DNA polymerase.

16S (Table 16): Amplification was performed in a thermocycler (Geneamp PCR System 9700). PCR conditions for COI and 16S consisted of an initial denaturation of 1.5 min at 94°C, followed by 35 cycles of 45s denaturation at 93°C, 1 min annealing at 50° C and 1 min extension at 72° C, followed by a final elongation of 5 min at 72° C. Amplification products were analyzed by electrophoresis on 2.0 % agarose gel in 1x TAE buffer at 100 volts for 1 hr. Gels were stained with ethidium bromide and immediately photographed under UV light. Sizes of bands were inferred by comparison with a 100 bp ladder (100 bp DNA Ladder, New England Biolabs) (deWaard et al. 2006).

18S (Table 16): Amplification were performed using the same thermocycler as 16S and COI but PCR conditions for 18S consisted of an initial denaturation of 1.5 min at 94° C, 35 cycles of 30s denaturation at 93° C, 30s annealing at 50° C, and 3 min extension at 72° C, followed by a final elongation of 5 min at 72° C. Amplification products were analyzed by the same procedure as for 16S and COI.

PCR purification

One unit of Shrimp Alkalane Phosphotase (SAP) and 3 units of Exonuclease I was added to the PCR product. PCR products were diluted to 100 µl with Tris/EDTA and another

100 µl of phenol was added. After vortexing, it was placed in a centrifuge (Eppendorf Centrifuge 5417R) for 10 min. at 10,000 rpm at 22°C. The aqueous top layer was placed in a new tube and 100 µl of phenol chloroform/isoamyl mix was added. It was again placed in the centrifuge for 10 min. One µl of glycogen was added to the aqueous top layer. Ten to twenty µl of sodium acetate was then added, depending on the final volume retrieved. Three hundred µl of 100% ethanol was added and the mixture was kept at -40°C for 5 min. After incubation, the mixture was spun at 10,000 rpm for 15 min at 4°C. The ethanol was poured off and 300 µl if 70% ethanol was added. The mixture was spun in the centrifuge again for 5 min at 4°C. The ethanol was poured off and mixture was spun in Spinvac for 5 min. Thirty µl of distilled water was added and PCR fragments were sent to Microgen in Korea to be sequenced (William Ferguson, personal communication).

Sequence Analysis

DNA sequences and compositions were aligned and compared using the program BioEdit Sequence Alignment Editor 7.0.9.0. Phylogenetic trees were also computed using the PHYLIP program. Intra- and inter-population analyses of gene diversity were performed using ARLEQUIN v. 3.5 (Kautenburger 2006, Martinez et al. 2006).

Such analyses include:

- Searching for shared haplotypes among populations
- Using AMOVA (Analyses of Molecular Variance) to evaluate the degree of population genetic structure
- Compute F-statistics and genetic distances between populations

- Test for selective neutrality within populations and deviations from Hardy-Weinberg equilibrium since parthenogenetic populations are heterozygote deficient.

RESULTS

RAPD analysis

Initial amplification tests revealed Primers 2 and 3 to show the most polymorphisms among individuals tested. Bands produced by primers 1, 4, 5 and 6 generated the same bands in individuals tested within puddles, among puddles, and among populations. Primers 2 and 3 also produced consistent amplification products in all individuals tested (Figures 39 and 40).

Using RAPD primer 2, four clones (2-A, 2-B, 2-C and 2-D) were found among puddles NJ 2, NJ 4, NJ 6 and NJ 8 in the year 2007 with clone A representing 85% of the clones detected among all puddles (Figure 39, Table 17). All individuals sampled from NJ 2 and NJ 4 were clone 2-A. Clones 2-B and 2-C were specific to NJ 6 and clone 2-D was specific to NJ 8. In NJ 6, clone 2-A represented 67% of the individuals, 11% were clone 2-B and 22% were clone 2-C. In NJ 8, clone 2-A represented 80% of the individuals and clone 2-D represented 20% of the individuals. In 2008, Clone 2-A reappeared in NJ 2 and NJ 6 and represented 90% and 70% of the clones found in each respectively. A new clone, clone 2-E appeared this year in both NJ 2 and NJ 6 and represented 10% and 30% of the clones found in each respectively (Figure 40). Clone 2-E represented 8% of the subpopulation of *C. gynecia*. A total of five clones was found among the puddles among

the 54 individuals sampled from the New Jersey study site during the years 2007 and 2008 (Tables 18 and 19). No individuals were collected from puddle 4 in 2008. Clone 2-A was also detected in both site locations in MA and comprised 80% of the individuals tested. Clone 2-F was found in Saugerties, NY and comprised 100% of the individuals. Clone 2-G was only found in Hyde Park, NY and comprised 100% of the individuals sampled there. Clone 2-H was found in Pittsfield, MA and comprised 20% of the individuals sampled.

In 2007, nine clones (3-A, 3-B, 3-C, 3-D, 3-E, 3-F, 3-G, 3-H1, and 3-H2) were found using primer 3 (Figures 42 and 43; Table 20). Clone 3-A represented 29% of the clones in NJ 2 and 44.5% of the clones in NJ 6 and comprised 17% of all clones found. Clone 3-B was found in puddle NJ 2, NJ 4, NJ 6 and NJ 8 represented 57%, 90%, 44.5% and 89% of the clones in each puddle, respectively. Clone 3-B also comprised 71% clones found that year. Clone 3-C was unique to NJ 2 and represented 14% of the clones found in the puddle and comprised 3% of all the clones found. Clone 3-D was only found in NJ 4 and represented 10% of the clones found in that puddle (3% of total number of clones). Clone 3-E was unique to NJ 6 and represented 11% of the clones found in that puddle (3% of total number of clones). Clone 3-F was unique to NJ 8 and represented 11% of the clones found in puddle 8 (3% of the total) (Tables 21 and 22).

In 2008, clone 3-A, 3-B and 3-C reappeared (Figure 44). Clone 3-A was found in NJ 2 and NJ 6, representing 57% (n=7) and 80% (n=10) of the clones found in each puddle respectively and comprised 71% of the clones found that year. Clone 3 B was also found

in NJ 2 and NJ 6 (14% and 20% respectively) and comprised 18% of the total clones found. Clone 3-C reappeared in NJ 2 and represented 14% of the clones found in the puddle and comprised 12% of the clonal population for that year. Clone 3-G was found in Saugerties, NY and represented 100% of the individuals sampled (n=10). Clone 3-H1 and 3-H2 was found in Hyde Park, NY and each clone represented 50% of the individuals sampled (n=4) (Figure 45). Clone 3-B was the only clone found at both collection sites in MA (Figure 46).

Combined analysis

Among the 66 individuals that were sequenced for both primer 2 and 3, the combination of the two revealed the presence of 18 clones within and among populations of NJ, NY and MA (Table 23). Clone A was found in all populations in both 2007 and 2008. Clone B was present in NJ 2 and NJ 6 in both 2007 and 2008. In 2007, clone C was unique to NJ 4. NJ 6 contained three unique clones – D, E, and F. NJ 8 contained four unique clones – G, H, I and J. In 2008, clones K and L appeared in NJ 2, clones M, N, O appeared in NJ 6, clones P and Q appeared in NY and clone R appeared in the MA population (Tables 24 and 25). Frequencies of each clone are found in Table 25.

Primer 3 produced more clones than Primer 2 half of the time. Primer 2 produced more clones than Primer 3 one quarter of the time. Combining both primers into a master data set increased the number of clones present in NJ6 (2007 and 2008) and NJ8 (2007). It provided an intermediate number of clones for NJ2 and NJ4 (2007). It produced the same

number of clones as Primer 3 for NJ2 (2008) and NY (2008) and produced the same number of clones as Primer 2 for MA (2008) (Table 26).

AMOVA results

When the results of RAPD primers 2 and 3 are analyzed separately, there was significant variation among populations (62.93% and 51.86%, respectively $P < 0.001$). When the results of the RAPD 2 and 3 are combined into a master set, there was significant variation found within the populations ($P < 0.0001$) (Table 27) inferring that individuals varied more within their respective puddles than across the different populations.

mtDNA sequencing

For the 16S fragment, ~350 base pairs (bp) were obtained in all individuals tested. In the 18S fragment, all individuals contained 613 bp. There were no differences in mtDNA sequences found among *C. gynecia* inhabiting the 10 puddles in New Jersey. No difference in mtDNA sequences were found among the NJ, MA and NY populations.

DISCUSSION

Clonal diversity among New Jersey sub-populations of C. gynecia

Repeated multilocus genotypes found among the genetic profiles of a population provides evidence of clonal reproduction, assuming there are at most two alleles per locus (one

dominant and one recessive) (Lynch and Milligan 1994, Halkett et al. 2005). RAPD analysis and mtDNA sequencing was used to test for the clonal structure within and among populations of *Caenestheriella gynecia*. Specific questions asked were: Are there different clones present in a given puddle found in the New Jersey population and if so, how many of each clone is present? Is there clonal diversity among the three populations of *C. gynecia* and do they share any clones?

The number of clones in each puddle varied depending on which RAPD primer was used. Primer 3 produced more (if not the same) clones per puddle than Primer 2 88% of the time (Tables 18 and 19). This questions the reliability of which primer could accurately determine the true number of clones in a puddle. For this reason, the remaining discussion will focus on the combined RAPD results as the combination of more than one molecular marker increases the chance of detecting differences among clone mates (Miermands and Van Tienderen 2004).

It is worth mentioning the results of a previous study on the detection of a virulent clone that causes serogroup C meningococcal disease using RAPD analysis. Schmink et al. (2001) found that differing primers were sensitive and specific enough to correctly identify the disease-causing clone. In other words, though they produced different numbers of clones (19 vs. 20, in this case), it is still an efficient way of determining the identity of clones. It may be possible to use RAPD primers as an initial identifier of a species of clam shrimp. In the future, RAPD Primers 2 and 3 could be tested across different species of clam shrimp to see if species-specific primers can be produced for use

in accurately identifying them as such. This would also allow for the rapid screening of a large number of individuals and ease the genetic identification of field samples. This gene-mapping method has proven to be successful in identifying seed and broodstock of tropical oysters in Thailand, for the red bread mold *Neurospora crassa* and the branchiopod *Daphnia pulex* (Jacobsen et al. 1996, Klinbunga et al. 2000, Colbourne et al. 2004). There are several advantages of gene mapping using RAPD markers. (1) Isolation of cloned DNA probes or nucleotide sequencing is not required. (2) RAPD primers are a set of universal primers that can be used for the genomic analysis in a wide variety of species and can facilitate the transfer of information in collaborative research programs. (3) RAPD analysis produces markers that are congruent in use to RFLP's (Restriction Fragment Length Polymorphism) but are easier to obtain (Williams et al 1990, Micheli et al. 1994, De Wolf et. al 1998, Sun and Wong 2001).

The combined RAPD results revealed the presence of more than one clone in puddles containing *C. gynecia* in their Meadowlands habitat. During the 2007-2008 sampling season, 15 clones were distinguished among the four puddles surveyed with Clone A shared among all. Clone B was present only in puddles 2 and 6. Both clones reappeared within their respective puddles in the second year of sampling. In addition to the shared clones, each puddle had their own unique clones that disappeared in the second year of sampling.

This phenomenon was also seen in other branchiopods. Studies on *Daphnia pulex* showed the presence/absence of one or more clones during different sampling years with

high clonal diversity among individual rock pools with up to 24 clones in a given pool in Hudson Bay, Manitoba. Out of 36 total clones found, there were 3 that were dominant among most pools although the presence of the other clones was dependent on the physicochemical variation (i.e., salinity, pH, and dissolved organic content) among the pools (Weider 1989, Wilson and Hebert 1992). In addition, there was a trend found that there are only a few clones that are widespread, while the majority of clones are restricted to one or a few habitats. These patterns were found to be related to whether the habitat was mesic (wet) or xeric (dry) (Weider 1989). Future studies on *C. gynecia* could test whether certain clones are unique to puddles which evaporate during a given time in their growing season. Another example of the effect of an organism's environment on its DNA can be seen in the Figueroa et al. (2002) study on the phenotype composition of the grain aphid *Sitobian avenae.* Changes in RAPD-PCR phenotypes were also observed among clones of the parthenogenetic grain aphid due to hydroxamic acids released from their host plant. Hydroxamic acid acts as a mutagen to the aphid's DNA and produced changes in the genetic material in as little as four generations of cultivating the aphids on wheat plants (9 generations on oat; 12 – 14 on barley).

Another explanation for the multiclonal genetic structure of *C. gynecia* is hatching of diapausing eggs found in the sediment of the puddles. De Meester et al. (2002) stated that resting propagules in sediment can amount to 10^4 to 10^6 propagules per square meter and of that amount only 10% of them respond to hatching stimuli at the start of a growing season. This leads to the appearance of different genotypes appearing at different points in the season. These eggs can remain dormant for more than 40 years and can hatch when

environmental conditions are favorable. When they do hatch, 'old' genotypes enter the gene pool of their present day descendants (Mattox and Velardo 1950, Colburne et al. 2004). This theory is supported with the differential hatching rates observed in the Life History section of this study. The MNJ population experienced at least three different onsets of hatching periods at 7, 14 and 21 days after hydration (Figure 14). This genetic 'storage effect' was also observed by Weider et al. (1997) after diapausing eggs of *Daphnia* sp. were isolated and hatched from sediment cores collected over a two-year period (1996-1997). These core samples possessed cysts that were 19 and 20 years old, respectively. Significant shifts in allelic composition were observed between the two years with the disappearance of one allele and the reappearance of another. This occurrence seemed to coincide with anthropogenic changes of *Daphnia's* aquatic habitat in Eastern Europe during that time period. Their data demonstrated that sufficient genetic variation was present in the cyst banks to allow natural populations to respond readily to changing selection pressures.

Asexual organisms living in small populations will eventually become extinct, as per Muller's ratchet, because of the accumulation of deleterious mutations (Normark et al. 2003)**.** This is combated in *C. gynecia* by the presence of diapausing eggs and cyst banks. This adaptation allows for the long term persistence of founder effects in which the population would be continually supplied with common genotypes due to variable hatching rates, even though new genotypes could emerge. However, these new genotypes may have lower fitness than the original founders and will not persist (McCafferty et al. 2010).

The multi-clonal genetic pattern observed in *C. gynecia* could also be the result of ingestion of foreign DNA due to desiccation affects. Bacterial, fungal and plant genes have been found in the telomeric regions of the Bdelloid rotifer. It appears to be due to horizontal gene transfer (the movement of genes from one organism to another other than direct descent) because of membrane disruption, DNA fragmentation and repair associated with repeated desiccation and rehydration (Gladyshev and Arkipova 2010, Gladyshev et al. 2008). Free DNA can make its way into the guts of aquatic invertebrates as it can be absorbed on mineral surfaces or bound by complex organic molecules found in aquatic and soil environments. As the gut and the ovaries of aquatic invertebrates are located within the same vicinity, it is possible that damage to the gut and ovary, experienced during desiccation, would facilitate the passage and ingestion of the foreign DNA. The results of this process may be mistaken to have arisen from sexual reproduction if conspecific DNA is reabsorbed at the same time. The foreign DNA could replace endogenous sequences by homologous recombination (Gladyshev and Arkipova 2010). *Caenestheriella gynecia* is a filter feeder like the bdelloid rotifer. The digestive tract was removed from individuals before DNA extraction in this experiment because of possible amplification of foreign DNA. However, multi-clonal RAPD patterns may have resulted if the DNA was already incorporated into the *C. gynecia* genome.

Clonal diversity among populations of C. gynecia

During the 2007-2008 sampling season, 18 clones were detected among the 66 individuals sampled among the three populations of *C. gynecia* with significant variation within each population. Combined RAPD results revealed that all populations of *C.*

gynecia possessed at least two distinct clones. However, only Clone A was found among all three. This suggests that Clone A may have been the founder genotype of the three populations which has a wide physiological tolerance of environmental conditions (as reported in the Ecological chapter) that increased their fitness in each habitat and allowed them to persist into the next year. Subsequent mutations occurring in each sub-population of *C. gynecia* would then produce the unrelated genotypes. This scenario was also inferred among populations of Chilean cereal aphids after low levels of genetic variation were found among populations of the obligate parthenogen suggesting different origins of introduction followed by genotypic diversification through mutations (Figueroa et al. 2005).

Only a small number of individuals were sampled from each subpopulation of *C. gynecia* and therefore it may be possible that all clones were not detected and represented in these results. Although RAPD results revealed clonal diversity among populations of *C. gynecia*, phenotypic diversity was not observed among individuals tested.

Lack of mtDNA variation

Several theories can explain the lack of differentiation among mtDNA sequences of *C. gynecia*, found in NJ. One theory is that new mutations in the clam shrimp's genome may not have accumulated to the point of detection as this takes a longer time to occur in strictly clonal mating systems than in their sexual counterparts (Fu et al. 1998, Halkett et al. 2005).

Another theory is that the wrong mtDNA sequence was selected to determine genetic variation in populations of *C. gynecia.* Sequence divergence for mitochondrial genes among crustaceans is highly variable but it also depends on the taxonomic level to which one is testing the variability. Selecting an appropriate gene for molecular comparisons among species require selection of a gene with sequence variation that match the taxonomic level of study (Glenn et al. 2002). Wetzer (2001) tested two ribosomal genes, 12S and 16S, and one protein-coding gene, COI, in isopods and found that sequence divergence increased among taxonomic levels with the smallest divergence occurring within and among populations (0 – 4.1%) to largest occurring at the family level (17.7 – 33.6%). This was observed for all three gene sequences. The outcome of the study was the suggestion that 12S is the most appropriate gene sequence (used cautiously) to use when studying variation among populations and species. In future studies, 12S mtDNA should be sequenced among different populations of *C. gynecia* to see if that sequence would show any variation.

There have also been explanations that suggest that the way a species came to be strictly clonal may influence their genetic diversity. Although a parthenogenetic species may contain multiple clones, the origin of genetic variation may be the result of mutations, recombination, or multiple hybridization events. If a species became clonal through repeated hybridization between sexual taxa then that species would become genetically diverse (Figueroa et al. 2005). Welch and Meselson (2001) found that the asexual Bdelloid rotifer genome possesses highly divergent copies of the *hsp82* gene with each copy found in separate ancient sexual lineages of the rotifer phylum. Chilean cereal

aphids are another example of a species that became an obligate parthenogen through hybridization. Evidence was provided as high levels of heterozygosity and low levels of genetic diversity were found among the Chilean population (Figueroa et al. 2005).

On the other hand, a spontaneous loss of sex at a given time followed by rapid colonization by clonal lineages would account for low genetic diversity (Welch and Meselson 2000, Halkett et al. 2005, Kearney 2005, Vorburger 2006). This is characteristic of organisms appearing after glacial maxima and supports Schmidt and Kiviat's hypothesis of the geographic distribution of *C. gynecia* due to the Wisconsinan Glacial episode which began about 30,000 years ago and ended about 10,000 years ago. They proposed that *C. gynecia* was derived from *Caenestheriella setosa* (a sexually reproducing species) whose habitat range is closest (Kearney 2005, Schmidt and Kiviat 2007, Schön 2007). Recently, Weeks et al. (2009) found, through phylogenetic analysis, that *C. gynecia* parthenogenesis was derived from a dioecious ancestor. However, they believe it was derived from *C. mexicanus* and not *C. setosa*. Although mtDNA sequencing of the 16S and 18S genes did not reveal any genetic variation among the different clones found within and among populations of *C. gynecia*, future genetic analysis using a combination of RAPD and 12S mtDNA gene sequencing should include representatives of the *C. mexicanus* and *C. setosa* to determine if *C. gynecia* originated from one or the other.

In addition, Schmidt and Kiviat (2007) proposed the following hypothesis on the dispersal and origin of *C. gynecia*: "*Caenestheriella gynecia* is derived from a sexually

reproducing species….dispersal into the glaciated area occurred in the Ohio Valley and was an unlikely event given the dispersal mechanisms of the species…..*C. gynecia* is relatively young (<12,500 years old)." This study revealed that *C. gynecia* is a strictly parthenogenetic species with no evidence of recombination (which is a signature of sexual species) (Barraclough and Herniou 2003, Halkett et al. 2005). MtDNA analysis, however, supports the notion that *C. gynecia* is relatively young as no genetic variation was observed within the NJ sub-populations or among the MA and NY populations. The number of substitutions that have accumulated during a certain time period in an evolutionary lineage is often taken as an alternative for the mutation rate (Ellegren 2009). As no substitutions were observed in the sequences amplified in *C. gynecia*, either *C. gynecia*'s mtDNA has an unusually slow rate of mutation or a recent (in evolutionary time) event occurred which caused a population bottleneck that reduced variation in the clam shrimp's genome followed by a range expansion (Glenn et al. 2002). As described above, *C. gynecia's* range expansion after the Wisconsinan Glacial episode can be such an event.

Direction of dispersal remains undetermined due to the lack of genetic variation among populations of *C. gynecia* found in NY, NJ and MA. Future studies should include samples from the first location of discovery, Oxford, OH, for this determination. The population genetic structure of colonizing organisms following a glaciation period should reflect high genetic diversity in populations in close proximity to the unglaciated area, with decreasing genetic diversity in populations as the distance from this refugia increase (Boileau and Hebert 1991). Enzymatic studies on the genetic structure of post-glacial

populations of the copepod, *Heterocope septentrionalis*, revealed that genetic diversity decreased in eastern populations of *H. septentrionalis* when compared to that of their western counterparts. Distances between populations ranged from 1 km – 30 km. Authors feel that the genetic differences may have occurred due to frequent bottlenecks causing genetic drift and reducing the genetic variation between populations (Boileu and Herbert 1991).

Similar results were seen in genetic comparisons of populations of the fairy shrimp *Eubranchipus vernalis*. High levels of genetic differentiation were observed in populations separated by >0.1 km. Geographically close populations (<0.1 km) of these branchiopods showed little genetic variation due to the effects of passive dispersal and limited gene flow (McCafferty et al. 2010). It was thought that the life-history of obligate aquatic invertebrates, with diapausing cysts, would allow for frequent dispersal due to wind and animals as prominent vectors. However, the authors suggest that priority effects (a term combining the persistent founder effects and monopolization hypothesis models of genetic drift) explain the genetic variation found within their study. Populations of *E. vernalis* are relatively young and experience rapid fluctuations in size giving them the inability to reach genetic equilibrium. Their large cyst bank also prevents immigrant genotypes from becoming established within the population because the variable hatching rates would lead to a consistent influx of the original genotypes (Boileau et al. 1992). It would interesting to see if 12S mtDNA sequencing would be able to elucidate genetic structure and the direction of dispersal for *C. gynecia* between the NJ, NY, MA

populations with distances between the three locations ranging from 77 km – 250 km (Figure 47).

Genetic differentiation as a result of dispersal via birds is another popular hypothesis used among ecologists and evolutionists alike (Santamaría and Klaassen 2002, Green and Figuerola 2005, Muñoz 2010). Waterfowl frequent lentic aquatic habitats (i.e., vernal pools, ponds etc.) more than lotic habitats (i.e., rivers and streams). The passive transport of aquatic invertebrates occurs when these waterfowl travel between the different habitats and ingest the propagules of aquatic invertebrates as they dabble in wetlands (Figuerola et al. 2005, Marten et al. 2006). Bird vectors can explain genetic differentiation among mtDNA for different forms of the branchiopod *Daphnia* found in North America, specifically *Daphnia ambigua* and *D. laevis* (Green and Figuerola 2005). Furthermore, their review indicates that, though waterbirds have great potential as dispersers of aquatic organisms, closely related bird species can have very different roles in dispersal of specific aquatic organisms, and great spatial and temporal variation is likely in dispersal patterns realized by a given bird population (Santamaría and Klaassen 2002). Migratory birds, i.e., Canada geese, and Great blue herons, have been observed in the pools inhabited by *C. gynecia*. Dispersal by migratory birds may well be one of the ways *C. gynecia* is being transported to its different locations.

To date, the ecological and evolutionary processes that mold genetic differentiation among wetlands in diapausing aquatic invertebrates are not understood. It has been hypothesized that human activity was the main force in transporting populations of *C.*

gynecia and could explain how and why populations are found in the northeastern states (Schmidt and Kiviat 2007). Anthropogenic activity has increased the natural dispersal rate of crustacean zooplankton up to 50,000-fold (Muñoz 2010). Some information that is lacking that would aid in studying these organisms are the roles of anthropogenic activities and large scale surveys on the distribution of aquatic invertebrate species (Muñoz 2010).

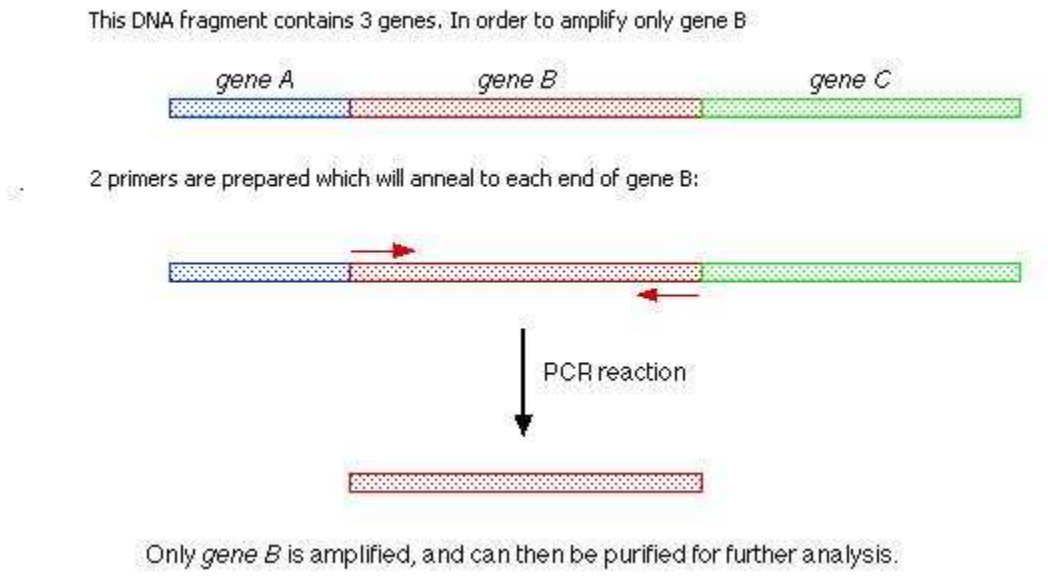


Figure 36. Standard PCR used to amplify a known sequence of DNA. An investigator chooses the sequence to amplify, then designs and makes primers which will anneal to sequences flanking the sequence of interest. www.avery.rutgers.edu

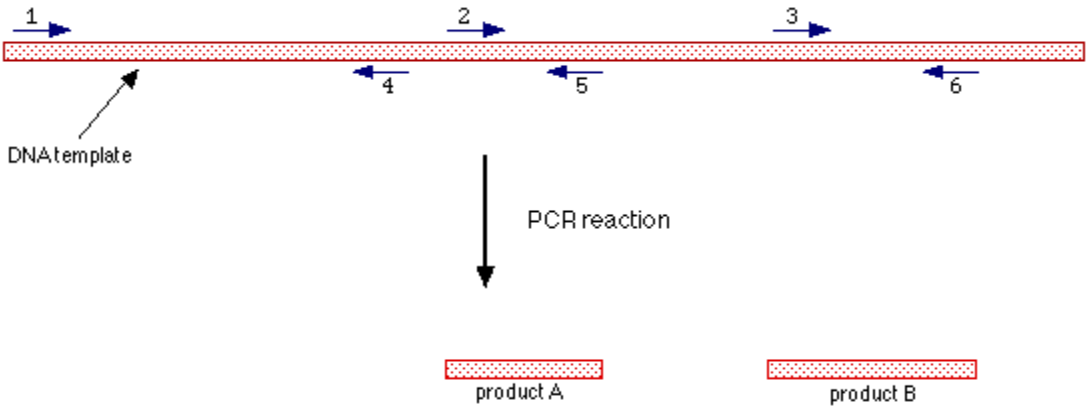


Figure 37. RAPD analysis with unknown target sequence(s). An arbitrary 10 base pair primer is synthesized and a PCR reaction is carried out. www.avery.rutgers.edu

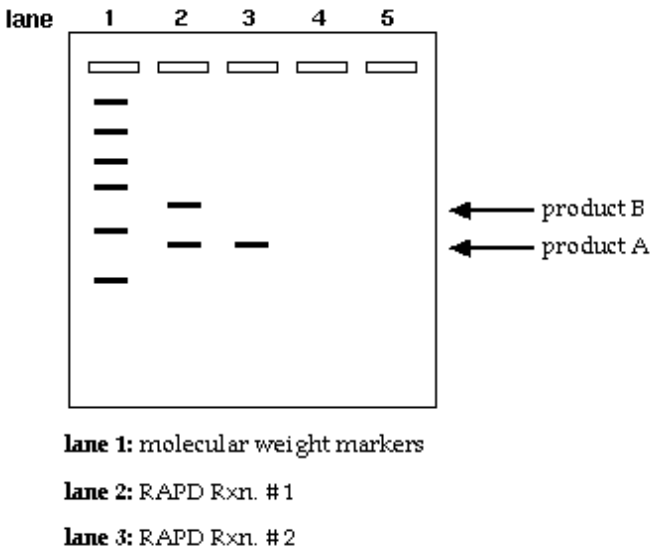


Figure 38. An agarose gel is run to see if any DNA segments were amplified in the presence of the arbitrary primer. (This gel represents two RAPD reactions with two different DNA templates, illustrating Figure 2 RAPD analysis in lane 2). www.avery.rutgers.edu

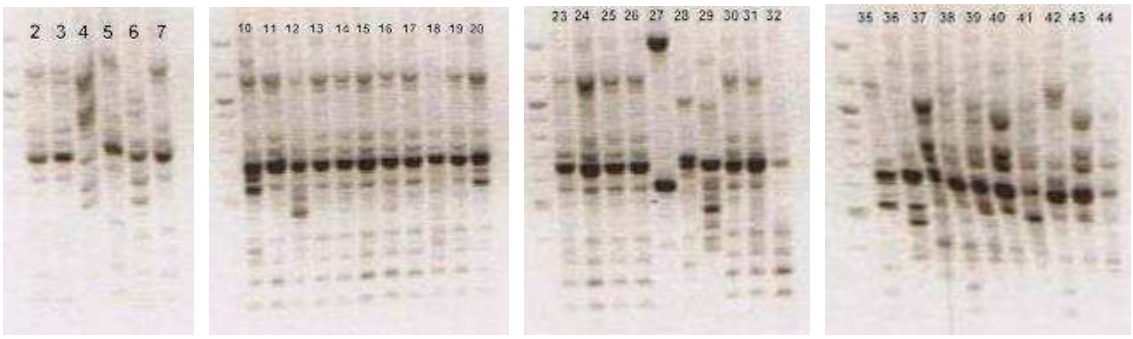


Figure 39. RAPD profiles for 37 individuals obtained using Primer 2. (Lanes 2-7): Puddle 2 individuals. (Lanes 10 -20): Puddle 4 individuals. (Lanes 23-32): Puddle 6 individuals. (Lanes 35-44): Puddle 8 individuals. All specimens were collected in 2007 in the Hackensack Meadowlands, NJ.

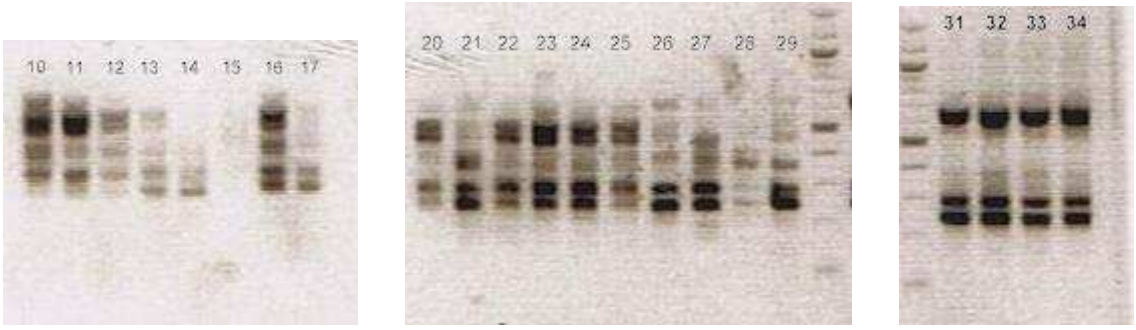


Figure 40. RAPD profiles for 21 individuals obtained using Primer 2. (Lanes 10-17): Puddle 8 individuals, Meadowlands, NJ. (Lanes 20-29): Larvae collected from rehydrated soil, Saugerties, NY. (Lanes 31-34): Individuals obtained from Hyde Park, NY. All specimens were collected in 2008.

Figure 41 RAPD profiles for individuals in NY and MA using primer 2. (Lane 1): 100 bpDNA ladder. (Lanes 2,3 and 6): Individuals from Pittsfield, MA. (Lane 4 and 5): Individuals from Lenox, MA. (Lane 7): Individual from Sugar Loaf, NY.

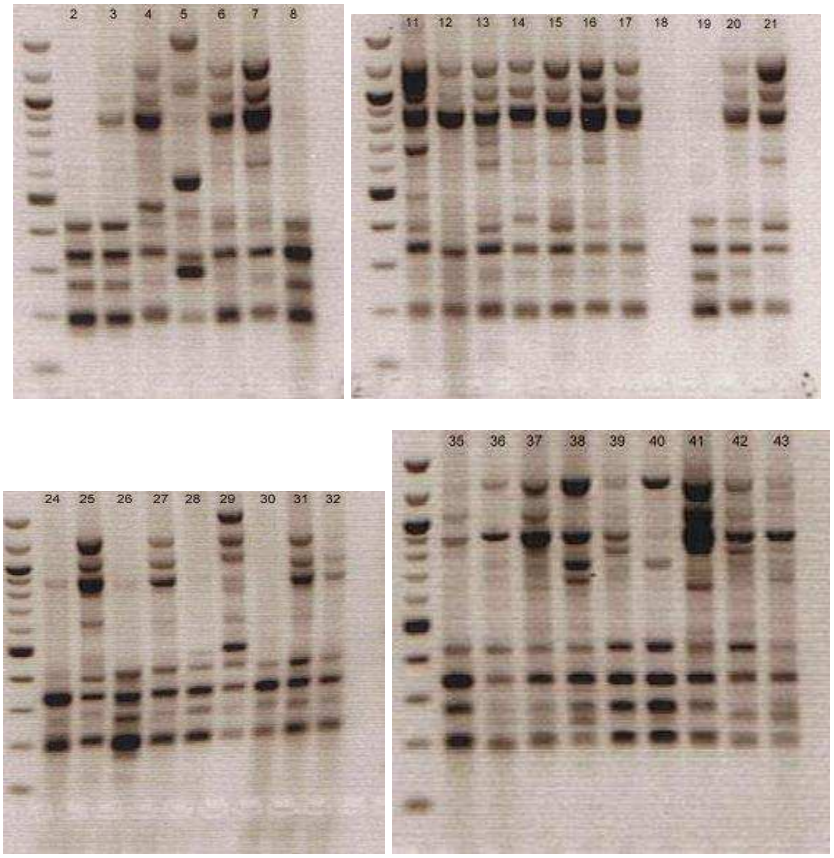


Figure 42. RAPD profiles for 35 individuals obtained using Primer 3. (Lanes 2-8): Puddle 2 (2007) individuals. (Lanes 11-21): Puddle 4 (2007) individuals. (Lanes 24-32): Puddle 6 (2007) individuals. (Lanes 35-43): Puddle 8 2007 individuals. All specimens were collected from the Meadowlands, NJ.

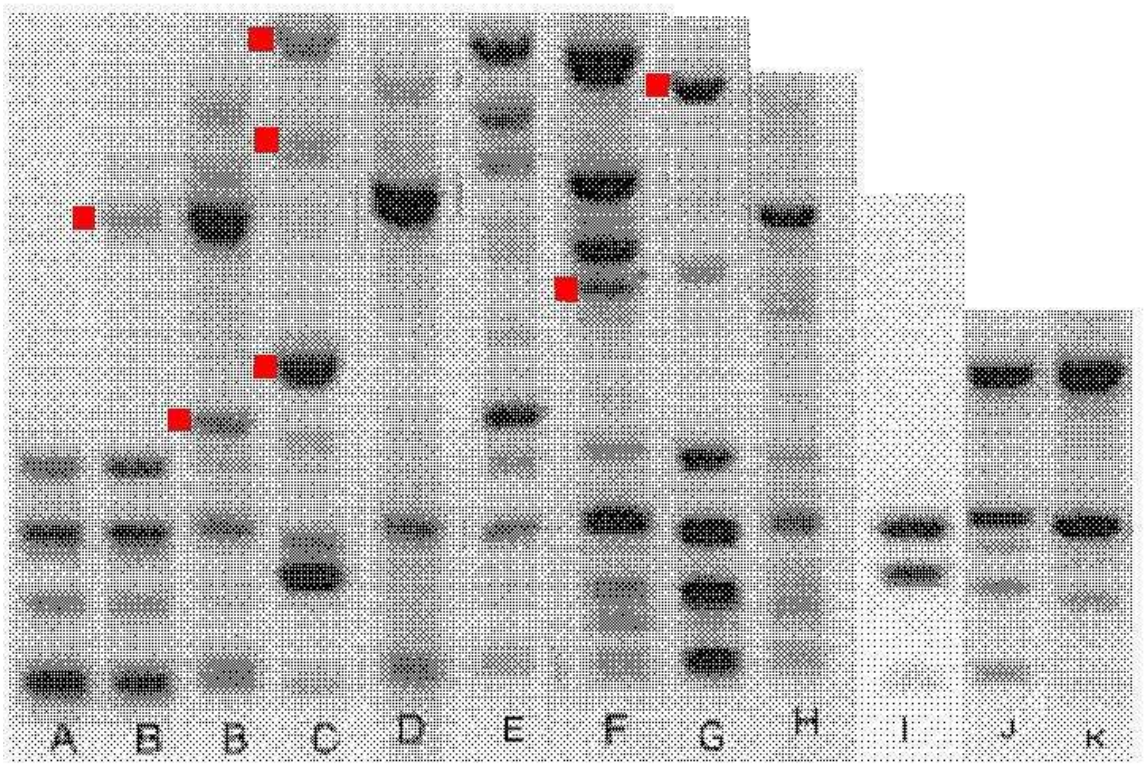


Figure 43. RAPD gel defining clones found with Primer 3. Red dots indicate which bands were considered to be polymorphic.

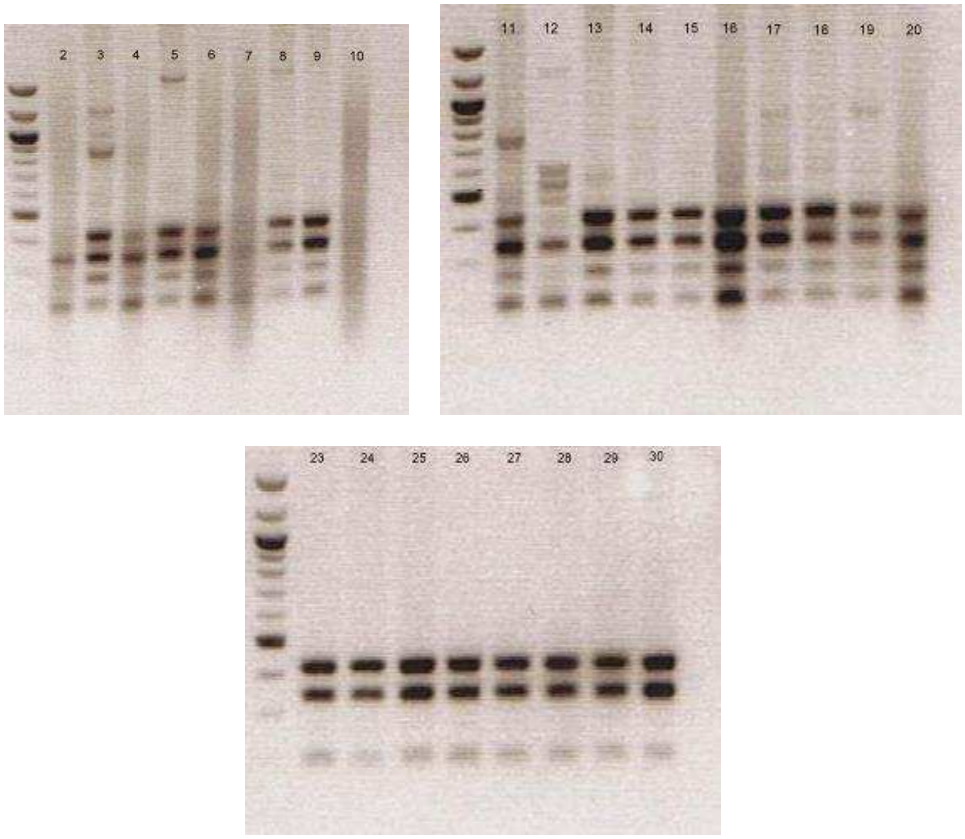


Figure 44. RAPD profiles for 35 individuals obtained using Primer 3. (Lanes 2-10): Puddle 2 individuals. (Lanes 11-20): Puddle 6 individuals. (Lanes 23 – 30): Puddle 8 individuals. All specimens were collected in New Jersey in 2008.

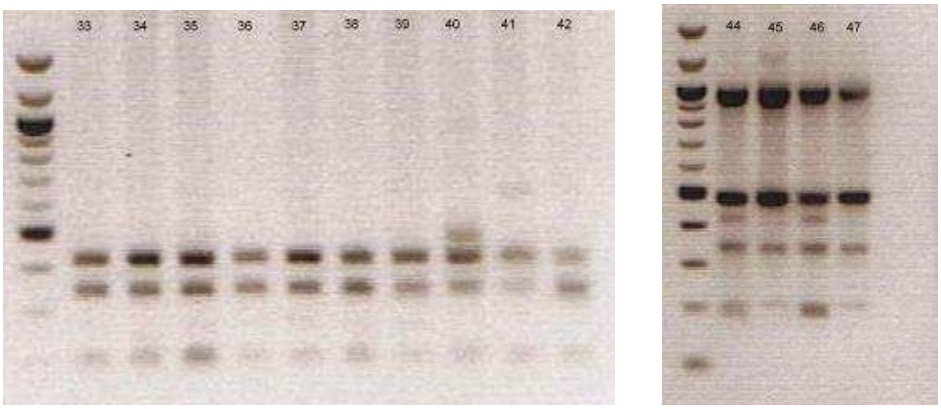


Figure 45. RAPD profiles for 14 individuals using Primer 3. (Lanes 33-34): Saugerties, NY. (Lanes 44-47): Hyde Park, NY. All specimens were collected in 2008.

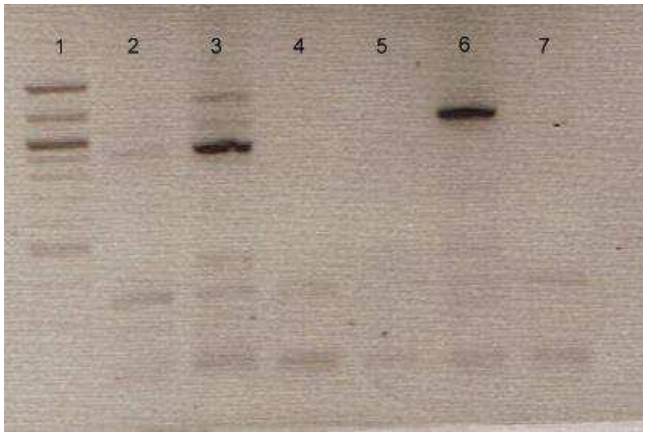


Figure 46. RAPD profiles for individuals in NY and MA using primer 3. (Lane 1): 100 bpDNA ladder. (Landes 2,3 and 6): Individuals from Pittsfield, MA. (Lane 4 and 5): Individuals from Lenox, MA. (Lane 7): Individual from Sugar Loaf, NY.

Figure 47. Aerial map of the 4 study sites and the original site of discovery for *C. gynecia* in Oxford, OH. A: MNJ; B: LPMA; C: SNY; D: SLNY; E: OH. Distances between: A:B=250 km; A:C=151 km; A:D=77 km; A:E=1038 km.

Table 15. RAPD analysis primers.

RAPD analysis primers	Primer sequence
Primer 1	5’ – d[GGTGCGGGAA] – 3’
Primer 2	5’ – d[GTTTCGCTCC] – 3’
Primer 3	5’ – d[GTAGACCCGT] – 3’
Primer 4	5’ – d[AAGAGCCCGT] – 3’
Primer 5	5’ – d[AACGCGCAAC] – 3’
Primer 6	5’ – d[CCCGTCAGCA] – 3’

Table 16. Primer sequences and PCR conditions.

Primers	16S	18S
Forward	5’-TGAACGGCTA AACGAGAAAA-3’	5’- TTAAGCCAT GCATGTCTAAG-3’
Reverse	5’-AGGTCGAACA GACCTTTTGT-3’	5’- CAACTACGAG CTTTTTAACC-3’
PCR cycling	1.5 min at 94°C, followed by 35 cycles of 45s at 93° C, 1 min at 50 C and 1 min at 72° C, followed by 1 cycle of 5 min at 72° C.	1 cycle of 1.5 min at 94° C, 35 cycles of 30s at 93° C, 30s at 50° C, and 3 min at 72° C, followed by 1 cycle of 5 min at 72° C

Table 17. RAPD 2 Clone definition: A – G: Absence /presence bands for base pairs – 1517, 1200, 800, 700, 650, 550, 500, 400, 300,250, 200, 100 (respectively).

Clone	Genotype
2-A	011111111011
2-B	100001000000
2-C	000011101011
2-D	000011111011
2-E	001000110100
2-F	000000111100
2-G	000010001100
2-H	100010111010

Table 18. RAPD 2: Frequency of clones in each puddle and population.

	2007				2008					
Haplotype	**NJ 2**	**NJ 4**	**NJ 6**	**NJ 8**	**NJ 2**	**NJ 6**	**Saugerties**	**Hyde Park**	**Pittsfield**	**Lenox**
n	(4)	(11)	(9)	(10)	(10)	(10)	(10)	(4)	(3)	(2)
2-A	4	11	6	8	9	7			2	2
2-B			1							
2-C			2							
2-D				2						
2-E					1	3				
2-F							10			
2-G								4		
2-H									1	

Table 19. RAPD 2: Relative frequencies of clones in each puddle and population.

	2007				2008					
Haplotype	**NJ 2**	**NJ 4**	**NJ 6**	**NJ 8**	**NJ 2**	**NJ 6**	**Saugerties**	**Hyde Park**	**Pittsfield**	**Lenox**
N	(4)	(11)	(9)	(10)	(10)	(10)	(10)	(4)	(3)	(2)
2-A	1	1	0.667	0.8	0.9	0.7			0.667	1
2-B			0.111							
2-C			0.222							
2-D				0.2						
2-E					0.1	0.3				
2-F							1			
2-G								1		
2-H									0.333	

Table 20 RAPD 3 Clone definition: A – J: Absence /presence bands for base pairs – 1517, 1200, 1100, 1000, 850, 750, 500, 400, 350, 300, 250, 200 (respectively).

Clone	Genotype
3-A	000000011011
3-B	010110011011
3-C	101000101101
3-D	010110001001
3-E	110100111011
3-F	010011011111
3-G	000000011001
3-H1	000100111001
3-H2	000100101001

Table 21. RAPD 3: Frequency of clones in each puddle and population.

		2007					2008			
Haplotype	**NJ 2**	**NJ 4**	**NJ 6**	**NJ 8**	**NJ 2**	**NJ 6**	**Saugerties**	**Hyde Park**	**Pittsfield**	**Lenox**
N	(7)	(10)	(9)	(9)	(7)	(10)	(10)	(4)	(3)	(2)
3-A	2		4		4	8				
3-B	4	9	4	8	1	2			3	2
3-C	1				2					
3-D		1								
3-E			1							
3-F				1						
3-G							10			
3-H1								2		
3-H2								2		

Table 22. RAPD 3: Relative frequencies of clones in each puddle and population.

		2007					2008			
Haplotype	**NJ 2**	**NJ 4**	**NJ 6**	**NJ 8**	**NJ 2**	**NJ 6**	**Saugerties**	**Hyde Park**	**Pittsfield**	**Lenox**
N	(7)	(10)	(9)	(9)	(7)	(10)	(10)	(4)	(3)	(2)
A	0.286		0.444		0.571	0.8				
B	0.571	0.9	0.444	0.889	0.143	0.2			1	1
C	0.143				0.286					
D		0.1								
E			0.111							
F				0.111						
G							1	0		
H1								0.5		
H2								0.5		

Table 23. Combined RAPD primer 2 and 3 Clone definition: Absence/presence bands for base pairs – First 12 loci correspond to primer 2 and last 12 loci correspond to primer3 base pairs.

Clone	Genotype	Clone	Genotype
A	011111111011010110011011	J	011111111011010001011111
B	011111111011000000011011	K	001000110100101000101101
C	011111111011010110001001	L	011111111011101000101101
D	100001000000010110011011	M	001000110100000000011011
E	000011101011000000011011	N	000010111100000000011011
F	000011101011110100111011	O	011111111011110100111011
G	000011111011010110011011	P	000000111100000000011001
H	011111111011010011011111	Q	000010001100000100101001
I	011111111011010010011111	R	100010111010010110011011

Table 24. Combined RAPD 2 and 3: Frequency of clones in each puddle and population.

	2007				2008			
Haplotype	**NJ 2**	**NJ 4**	**NJ 6**	**NJ 8**	**NJ 2**	**NJ 6**	**NY**	**MA**
n	(4)	(10)	(8)	(9)	(7)	(10)	(13)	(5)
A	3	9	2	4	1	2	1	4
B	1		3		4	4		
C		1						
D			1					
E			1					
F			1					
G				1				
H				1				
I				2				
J				1				
K					1			
L					1			
M						1		
N						2		
O						1		
P							8	
Q							4	
R								1

Table 25. Combined RAPD: Relative frequencies of clones in each puddle and population.

	2007				2008			
Haplotype	**NJ 2**	**NJ 4**	**NJ 6**	**NJ 8**	**NJ 2**	**NJ 6**	**NY**	**MA**
n	(4)	(10)	(8)	(9)	(7)	(10)	(13)	(5)
A	0.75	0.9	0.25	0.444	0.143	0.2	0.0769	0.8
B	0.25		0.375		0.571	0.4		
C		0.1						
D			0.125					
E			0.125					
F			0.125					
G				0.111				
H				0.111				
I				0.222				
J				0.111				
K					0.143			
L					0.143			
M						0.1		
N						0.2		
O						0.1		
P							0.615	
Q							0.308	
R								0.2

Table 26. Number of clones produced in each population by Primers 2 and 3 and by the combination of the two.

Population	**Year**	**Primer 2**	**Primer 3**	**Combined**
NJ2	2007	1	3	2
	2008	2	3	3
NJ4	2007	1	3	2
	2008	-	-	-
NJ6	2007	3	2	5
	2008	2	2	5
NJ8	2007	2	2	4
	2008	-	-	-
NY	2008	2	3	3
MA	2008	2	1	2
	Total	8	9	18

Table 27. Results of the AMOVA testing for significant differences among sub-populations of *C. gynecia* using RAPD results from primers 2, 3 and a combination of both (*P* = 0.0000).

	Primer 2	Primer 3	Combined
Among population	62.93	51.86	44.78
Within population	37.07	48.14	55.22
FST index	0.62926	0.51858	0.44782

CHAPTER VI: MATING SYSTEM OF *CAENESTHERIELLA GYNECIA*

INTRODUCTION

Current evolutionary theory proposes that sexual reproduction is nearly universal and purely asexual species appear to suffer early extinction (Milius 2003, Normark et al. 2003). Many hypotheses attempt to explain why sexual reproduction arose and persisted through time. When offspring production (number, development and survival) is deemed equal between the two modes of reproduction, a rare mutation in an obligate parthenogenetic species should spread and eliminate the sexual population (Lively and Johnson 1994, Corley and Moore 1999). Contrastingly, there are many scientists who still wonder why there are still asexual organisms that exist without the numerous benefits that come from reproducing sexually. The search for selective advantages to sex and recombination has gone on since its initiation in the 1930s by Fisher and Muller (Charlesworth 2006).

One thought on the origination of asexual organisms is that they are descendants of sexual lineages that never reversed back to the sexual mating system. Asexual reproduction may have developed when organisms were faced with extreme environmental conditions that prevented sexual reproduction from occurring (Corley and Moore 1999). Evolutionary biologists argue that it would require more evolutionary steps to reacquire sexual reproduction than it does to lose it. Once sex has evolved, it may be difficult to return to a form of asexual reproduction due to developmental constraints such as difficulty in switching from meiotic to mitotic reproduction and/or from ploidy

differences or mutation accumulation (Corley and Moore 1999, Bode et al. 2010). Another assumption is that reversals are unlikely because mutations in loci essential for sexual reproduction accumulate in asexual lineages, therefore making it difficult to revert back to a sexual mating system (Normark et al. 2003, Bode et al. 2010).

Disadvantages of asexuality include the lack of genetic variation needed for evolutionary change because organisms are unable to outcross. An increase in the number of mutations that can accumulate in an asexual organism's genome may also result from the lack of recombination without sex and can lead to extinction (Bode et al. 2010). Sex and recombination "corrects" this by combining favorable mutations and avoiding the accumulations of deleterious ones. The DNA in offspring of sexual organisms undergoes many gene conversions and crossovers causing the individual's chromosomes to become a mosaic of maternal and paternal materials (Corley and Moore 1999, Charlesworth 2006). In contrast, the DNA of offspring resulting from asexual reproduction show little to no variation from their parental origins. The reduction in genetic variation can lead to a reduced tendency for a species to adapt to environmental changes. Asexual organisms are therefore short-lived in evolutionary terms in comparison to their sexual relatives (Bode et al. 2010). When compared to their sexual counterparts, asexual species have a lower lifetime fecundity and fitness, slower development of both the offspring produced and the adults as they matured (Corley and Moore 1999).

Despite the many disadvantages, there are a few species of animals or plants that reproduce only asexually. There are some benefits to being an asexual organism. Asexual

lineages can increase their numbers rapidly because all members are female and all can produce viable eggs. Thus, only one female is necessary to colonize an area if presented with ideal conditions, especially isolated habitats. The overproduction of zygotes in asexual individuals increases the number of viable progeny. Additionally, asexuals have a twofold fitness advantage over sexual lineages as individuals that reproduce asexually do not have to produce males and are therefore able to produce twice as many female offspring (Lively and Johnson 1994, Corley and Moore 1999, Charlesworth 2006). Another potential benefit, which can also be a cost of asexual reproduction, is the identical genetic makeup of the offspring and its parent. This genetic similarity may be beneficial if the genotype is well-suited to a stable environment (Barraclough and Herniou 2003, Simon et al. 2002).

One aquatic invertebrate group that exhibits parthogenesis is the bdelloid rotifers. Bdelloidea contains 360 species that have evolved without sex for at least 40 million years. They are the largest metazoan taxon in which males, hermaphrodites, and meiosis are unknown (Welch and Meselson 2000). The ovaries of bdelloid rotifers develop eggs containing the full genome of the species, in contrast to sexual organisms whose gonads split the genome in half when making eggs and sperm. Interestingly, the bdelloid species show more genetic variability among one another than genes that have evolved in sexually reproducing rotifers. This occurs because of the lack of reshuffling of genes that occur during sexual reproduction. As a result, the genes had plenty of time to build up mutations. Like for the clam shrimp *C. gynecia,* no males have been demonstrated to be present (Milius 2003).

Darwinulid ostracods are another extant taxon of asexual crustaceans. They range widely throughout salt and fresh water and include lineages that do have sex, although a systematic review of fossils shows no evidence of males for at least 200 million years (Milius 2003). Darwinulid ostracods show a different genetic pattern, with extreme uniformity within the asexual species, instead of bdelloid's genetic variation.

In recent years, there has been an increase in the use of species within the Branchiopoda class to study the evolution of mating systems. This class has representatives of the most common mating systems: dioecy (males and females within a population), androdioecy (males and hermaphrodites), hermaphroditism (individual possessing both male and female reproductive organs), parthenogenesis (development of embryos without fertilization in a female), and cyclical parthenogenesis (many rounds of parthenogenesis with a single episode of dioecy) (Rispe and Pierre 1998, Taylor et al. 1999, Zucker et al. 2001, Simon et al. 2002, Berg 2005, Weeks et al. 2009). This enables scientists to examine the origination of asexual reproduction from sexual ancestors and/or the reverse.

Conchostracans have representatives of most types of mating systems. Cyclical parthenogenesis can be seen in *Cyclestheria hislopi. Limnadia lenticularis* is a self compatible hermaphrodite (Weeks et al. 2005). The least common mating type is androdioecy, which is a mixed mating system, composed of males and self-compatible hermaphrodites (Weeks and Zucker 1999, Zucker et al. 1997, Otto et al.1993, Sassaman and Weeks 1993, Zucker et al. 2001). To date, the mating system of the conchostracan shrimp *Eulimnadia texana* has been extensively studied. There are three mating types

within this species consisting of *ss* males, *Ss* hermaphrodites (amphigenic), and *SS* hermaphrodites (monogenic). The *s* and *S* alleles are inherited as alternative genetic elements, with *s* recessive to *S* in determining sex. Both *Ss* and *SS* hermaphrodites are capable of selfing and outcrossing with males but neither can fertilize the eggs of others because they lack the clasping appendages to do so. *Ss* hermaphrodites produce all three types of offspring, including males, where as *SS* can only breed true during selfing (Weeks and Zucker 1999, Sassaman and Weeks 1993, Otto et al. 1993).

Unlike *E. texana, C. gynecia* is a parthenogenetic conchostracan with no record of males. This form of reproduction occurs naturally among invertebrates (aphids, daphnia, and rotifers) and vertebrates (reptiles, fish, amphibians and some birds) (Savage 2005). It does not occur in mammals because of their imprinted genes. Genomic imprinting inactivates some genes in either parentally derived genomes so that no one parental genome is imprinted twice. In order for successful development of a zygote, both a maternal and a paternal set of chromosomes need to be present (Taylor et al. 1999, Charlesworth 2006). In a few non-mammalian species it is the only method of reproduction, but more commonly occurs only at certain times, e.g., cyclical parthenogenesis in aphids (Milius 2000, Simon et al. 2002, Normak et al. 2003).

Morphological observations used to be the primary method in detecting clonality among species and were limited to taxa with sexual dimorphism (Halkett 2005, Muñoz 2009). There are many limitations to this approach. Sexual recombination cannot be implied from the presence of sexual forms as they may be relicts of sexual ancestors and might

not be functional (Milius 2003, Halkett 2005, Charlesworth 2006). There are also many facts that explain why there is a lack of sexual dimorphism in a species. It might result from the fact that males are rare, may be produced under different environmental conditions than the one being studied, or they can be morphologically too different to be recognized as such (Halkett 2005).

By looking at the genetic structure of *C. gynecia* among puddles found in Meadowlands, NJ, it should be possible to determine their true mating system. If *C. gynecia* are truly parthenogenetic, the "mother's" DNA should be identical to all "daughter" offspring. If not, then morphological comparisons among specimens should reveal one or both of two possible explanations. First, if modified appendages are found, there are males present in the population, indicating that they are androdioecious. Second, if ovaries and testis are found in any one individual, it further supports androdioecy with hermaphrodites as the majority and male clam shrimp as the minority.

All other American species of *Caenestheriella* have males and *C. gynecia* is the only one of three species of conchostracans in the world in which males are not yet known (Emberton 1980). Combining genetic, morphological and ecological information can provide insights on mating systems and species determination as compared to using investigations based on morphology alone (Muñoz 2009). In this study, I investigated the null hypotheses that *C. gynecia* is an androdioecious species with males present. In this section, I interpret the results of the morphological investigations and RAPD results to determine the possible mating system of *C. gynecia.*

RESULTS AND DISCUSSION

No males were found among the 363 individuals examined in this study. During the 2007-2008 sampling season, 15 clones were detected among the NJ population of *C. gynecia* and 18 clones were detected among the 66 individuals sampled among the three populations of *C. gynecia* with significant variation within each population. RAPD results revealed that all populations of *C. gynecia* possessed at least two distinct clones. However, only one clone (Clone A) was found among all three. This suggests that Clone A may have been the founder genotype of the three populations which has a wide physiological tolerance of environmental conditions (as reported in the Ecological chapter) that increased their fitness in each habitat and allowed them to persist into the next year. No differences in mtDNA sequences were found among *C. gynecia* inhabiting the 10 puddles in New Jersey or between the NJ, MA and NY populations.

It was recently suggested that *C. gynecia* is a hermaphroditic clam shrimp with rearing and histological data to support this hypothesis (Stephen Weeks, University of Akron, unpublished data). Weeks et al. (2005) outlined procedures for determining the mating system in clam shrimp (Figure 48). It is a multi-step process which involves the raising and isolating ‘‘female’’ clam shrimp, raising their offspring, determining sex ratios, determining genetic patterns and finally confirming or refuting the presence of ovotestes in the gonad region. If viable offspring are produced during isolation, then the “females” are either parthenogenetic females or self-compatible hermaphrodites. Next steps would involve establishing what type of female the clam shrimp is by observing the sex ratios of the offspring produced, as one that producing 25% males would be considered

amphigenic (produce hermaphrodites and males) and ones that have no males would be considered monogenic (can only produce hermaphrodites) (Sassaman and Weeks, 1993).

Weeks et al. (2005) believe that the most parsimonious explanation for finding any amphigenic hermaphrodites among the surveyed clam shrimp is that the species is androdioecious. Conversely, lack of amphigenics can either be because the species is parthenogenetic or because the species is androdioecious but the sample population surveyed is comprised of only monogenic hermaphrodites. Genetic analysis and sectioning of the gonad would be necessary to differentiate between these last two alternatives (Zucker et al. 1997). It was hard to determine the type of females found in the MJ population due to unsuccessful attempts to rearing *C. gynecia* until maturity in the lab (as reported in the Life History chapter). Future studies on the mating system of *C. gynecia* should include a morphological study using both the transmission electron microscopy (TEM) and scanning electron microscopy (SEM) on tissue sections of the gonads in populations found in NJ and genetic analysis of the offspring of *C. gynecia* "females" (Scanabissi and Mondini 2002).

In a study using a combination of sequence data for 28S rDNA, the elongation factor 1-alpha (EF1α) and cytochrome *c* oxidase I (COI) to outline breeding system transitions in the Limnadiidae family of conchostracans, Weeks et al. (2009) deduced that the parthenogenesis observed in *C. gynecia* may have been derived from the dioecious species of clam shrimp *Cyzicus mexicanus* as a result of a mutation which may have suppressed meiosis in *C. mexicanus*. The tadpole shrimp, *Triops* sp., is another

branchiopod in which histological studies revealed testicular lobes within the ovaries suggesting that they are anatomically self-compatible hermaphrodites. However, this physiological observation does not confirm hermaphroditism because there was no direct cytogenetic evidence for the restoration of diploidy by the haploid oocytes produced (Obregón-Barboza et al. 2001).

Limnadia lenticularis was another clam shrimp previously thought to be strictly parthenogenetic, with no record of males. Progressive studies proved otherwise. Tinti and Scanabissi (1996) found *L. lenticularis* to have low levels of genetic variation after allozyme analysis. After reinvestigation of museum samples of *L. lenticularis,* a small number of males (4 out of 364) were discovered in Austria (Eder et. al 2000). Scanabissi and Mondini (2002), using TEM and SEM, observed the possibility of self fertilization in *L. lenticularis* as testicular tissue was found in its gonads and the presence of spermatozoa within the same gonadal region as where the oocytes were descending. Again, future genetic analysis of *C. gynecia* should sequence 12S mtDNA among the different populations and progeny of *C. gynecia* to see if genetic variation would be revealed. Wetzer (2001) showed 12S mtDNA was able to reveal variation among populations and species of an isopod taxon. A population of all hermaphrodites should be expected to be highly homozygous and have low levels of allelic diversity. A parthenogenetic species would be expected to have high levels of heterozygosity as heterozygous alleles would be fixed in the parents and offspring. Heterozygosity should accumulate over time as the alleles at a given locus can mutate and diverge over generations (Normark et al. 2003, Weeks et al. 2005).

In conclusion, the population of *C. gynecia* has by low genetic variability based on the results obtained through RAPD and mtDNA sequence analysis. The lack of males within *C. gynecia's* population and low levels of genetic variability support the clonal nature of a strictly parthenogenetic species who seem to have ecological success in areas populated as there were some clones present throughout the two-year study.

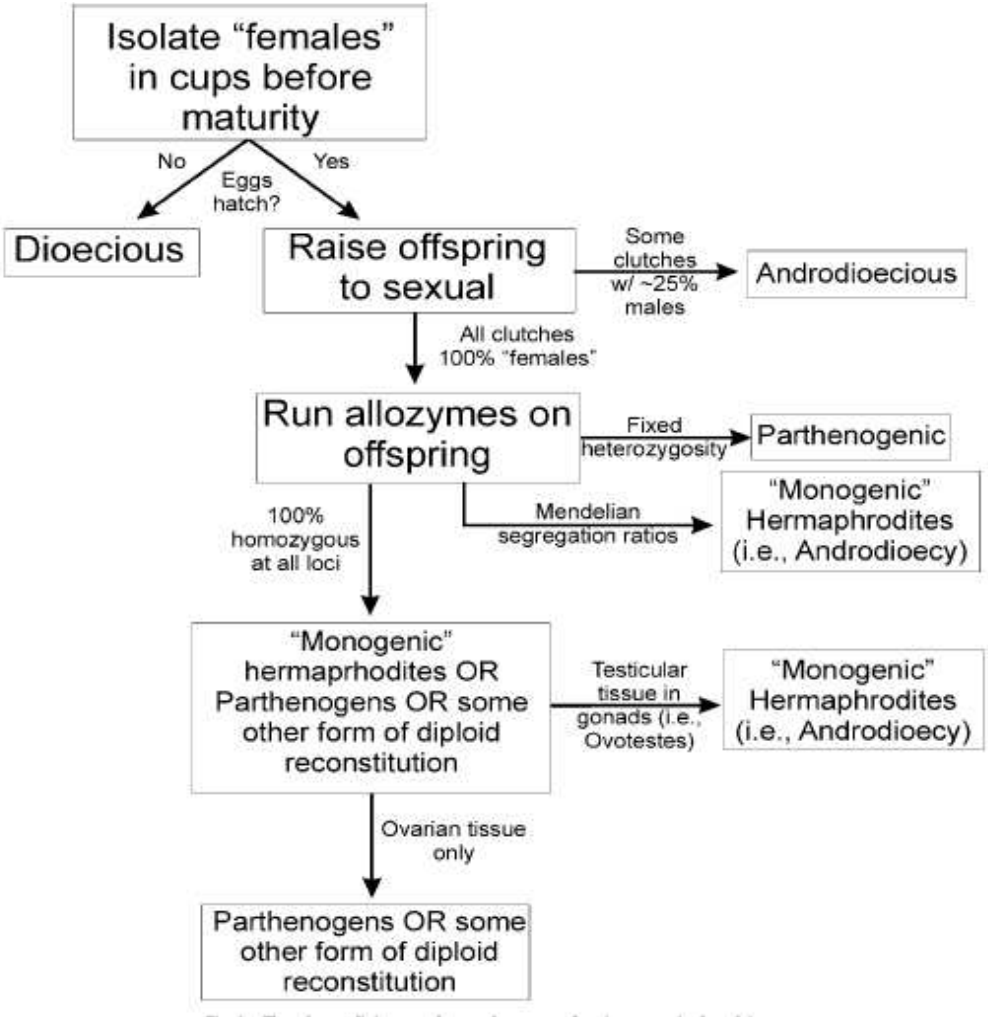


Figure 48. Flow chart from Weeks et al. (2005) outlining procedures to detect type of mating system in clam shrimp.

CONCLUSIONS

CONSERVATION OF *CAENESTHERIELLA GYNECIA* HABITAT

As development increases across North America, so does the number of urban, industrial and altered landscapes. Although these environments are being "redesigned" for human use there are certain species that can survive or even flourish in human-altered landscapes. Some of the focus of research and conservation should shift away from wildlands and natural areas and veer towards habitats created from human activities. Like natural and semi-natural habitats, altered habitats known to sustain rare organisms should be surveyed routinely and should be considered for conservation management.

ECOLOGY AND CONSERVATION

Vernal pools are a valuable and increasingly threatened ecosystem. The unique environment of vernal pools provides habitat for numerous rare plants and animals that are able to survive and thrive in these harsh conditions (Nicolet et al. 2004, Eitam et al. 2004). Vernal pools formed by anthropogenic factors are often overlooked in biodiversity assessment and monitoring schemes due to their small size and isolated nature (Nicolet et al. 2004). Consequently, vernal pools are often filled or drained as the surrounding uplands are developed. Human development and land use practices may also degrade the pool's water quality or alter its physical characteristics often changing its original function, thereby not allowing them to meet certain criteria necessary to be considered a functional vernal pool (Lathrop et al. 2005).

The Environmental Protection Agency lists the following four defining biological and physical criteria in their guide to seasonal pools (2005):

- **Hydrologic Isolation –** No permanent surface water connections to other water bodies. Some pools can be completely surrounded by terrestrial environment with the nearest aquatic habitat a half-mile or farther away, while other pools may occur within a larger wetland complex.
- **Periodic Drying –** Water levels generally fluctuate by season; pools experience drying or lowered water levels on a regular basis (frequency ranges from every year to just drought years).
- **Small Size and Shallow Depth –** Small area and shallow depth compared to other productive aquatic habitats (such as lakes and types of wetlands).
- **Distinctive Biological Community –** Support animals that are adapted to seasonal pool drying; support the breeding of animals that reproduce optimally without fish populations; do not support permanent populations of predatory fish.

The New Jersey Department of Environmental Protection (NJDEP) extends the periodic drying criteria to include that the pool must also maintain ponded water for at least two consecutive months between March and September. The pool must also be known and documented for supporting an obligate vernal pool breeder (cannot use any other habitat for breeding) or several facultative breeders (uses vernal pools but can be found to use other types of habitats) (Lathrop et al. 2005).

The results of my study indicate that the vernal pools located in Meadowlands, NJ are dynamic with a wide variation of temperature, water level, and other environmental conditions. The reference populations of wetlands considered in my study represent an assemblage of vernal pools in New Jersey with numerous small, shallow pools and relatively few deep, high-volume pools. The pools meet the NJDEP criteria as they do not have a permanently flowing outlet, most are found to maintain water for more than two consecutive months in each of the sampling years (April to November), they range in depth up to only a few centimeters (deepest was 65 cm), and they support a designated "At Risk" invertebrate clam shrimp species, *C. gynecia*. To further complicate conservation of these pools, there are likely to be several hundred un-documented pools scattered across NJ because many occur on private property with limited access, such as the Kane Tract.

LIFE HISTORY AND CONSERVATION

Branchiopods are usually the most conspicuous inhabitants of temporary waters because of their relatively large size and particular adaptations to the transient character of their habitat. The desiccant resistant eggs produced by *C. gynecia* allow the species to adapt to temporal environments and allow for passive dispersal by wind, rain, aquatic insects or migratory birds. This form of dispersal may lead people to expect that *C. gynecia* is a cosmopolitan species and can be found in many different regions of the United States.

Despite *C. gynecia* being passive dispersers, migration among puddles is possible during periods of increased precipitation. However, during periods of drought, connectivity is

decreased. If clam shrimp exhibit a metapopulation structure, they require recolonizations for long-term population viability. Populations will be lost if inter-puddle movement is prevented and there is a reduced likelihood of recolonization events following population extinction. To ensure the survival of the Meadowlands *C. gynecia* population, their habitat must be maintained in close to its present condition.

Possible conservation actions for the vernal pool habitats, as suggested by Schmidt and Kiviat (2007), may include controlled motor vehicle activity which will help to maintain the depth, size and turbidity of the puddle. This form of maintenance does not come without any costs, though, as the cysts of branchiopods are vulnerable to crushing by off-highway vehicles (OHV). Hathaway et al. (1996) found that very small forces, < 1 newton, can crush the cysts of the anostracan, *Branchinecta mackini* and the notostracan, *Triops longicaudatus*. The cysts are especially vulnerable when wet. It is very important to take caution toward OHV traffic particularly during the wet seasons as the cyst banks of *C. gynecia* can be found within the top few centimeters of soil.

The occurrence of *C. gynecia* is irregular in space and time and the hatching of their dormant eggs depends on a combination of the highly variable local climatological, hydrological conditions and environmental characteristics, such as temperature. These are important hatching triggers because they are reliable indicators of the suitability of the current inundation necessary for their growth and reproduction (Schönbrunner and Eder 2006, Day et al. 2010). *Caenestheriella gynecia* should therefore be considered an endemic species unique to the Northeast region of the United States.

There are two subcategories of endemism - paleoendemism and neoendemism. Paleoendemism refers to a species that was formerly widespread but is now restricted to a smaller area. Neoendemism refers to a species that has recently arisen such as a species that has diverged and become reproductively isolated, or has formed following hybridization and is now classified as a separate species (Kraft et al. 2010). *Caenestheriella gynecia* may be neoendemic as fossil *Cyzicus* clam shrimp have been found in Triassic fresh water deposits in Granton Quarry in North Bergen, New Jersey. *Cyzicus* fossils have also been found Weehawken and Princeton, New Jersey (Olsen 1980). Results on the morphology of the different populations of *C. gynecia* within my study support neoendemism as clam shrimp populations of New York are considered to be *Cyzicus* and not *Caenestheriella*, as once believed (Morphological chapter). However, based on the lack of mtDNA variation found in the genetic analysis of the different populations, it is difficult to determine where and how long ago *C. gynecia* evolved. Other clam shrimp found to be endemic to their localities are *Cyzicus grubei* and *Maghrebestheria maroccana.* They inhabit temporary pools ranging from central to southwest Portugal on the Iberian Peninsula (Machado et al. 1999).

To successfully determine the area of endemism of *C. gynecia*, it can be suggested to follow a protocol reiterated by Morrone (1994) in which its range would be mapped based on the combination of the following criteria: where the species ranges are relatively small compared with the whole region itself; where their distributional limits are accurately known; and the validity of the species is not in dispute. Resulting overlaps in distribution ranges would determine an area of endemism for the species.

Once the area of endemism is determined, the habitat in which a species is found should be managed to keep it in its most natural state. Nicolet et al. (2004) suggests some management steps for temporary pools that can be transferred to the vernal pool habitats in which *C. gynecia* are found. The first step includes extensive surveying of the temporary ponds to assess their conservation value, including the presence of uncommon species. Another necessary step is to monitor and protect the hydroperiod characteristics of temporary ponds. The final step would be the creation temporary pond mosaics that can be done on relatively small areas of land. This would serve to replace any habitat lost due to anthropogenic factors, i.e., construction, filling in of wetland. My study provides valuable information that can be used in the management of *C. gynecia's* vernal pool habitat. It is the first extensive survey of *C. gynecia's* habitat in New Jersey, and provides insight on the numerous wildlife that relies on their habitats. It can also serve as a two-year review of the hydroperiod dynamics of the temporary puddles.

Conservation efforts tend to focus on large charismatic species as they often require large areas to be set aside so that all their needs are met (i.e., finding mates, gathering food, etc.) (Belk 1998). It is usually assumed that the smaller species, such as invertebrates, that inhabit the same area will be conserved as well (termed the umbrella species concept (Poiani et al. 2001, Suter et al. 2002). Belk (1998) shares similar ideas when he states that if one was not studying a particular taxa, then others would not become aware that the habitat, and other species within it, needs protection. The results of Kerr (1997) negate this concept as his study showed that targeting mammals for conservation does not in turn conserve centers of invertebrate species richness and is likely to lead to inadequate

biodiversity protection. It would therefore be necessary to designate smaller areas for conservation based on invertebrate distribution, especially in urban areas where their habitat is under constant degradation.

The Kane Tract of New Jersey would be a perfect example of an area needed to be conserved for the conservation of *C. gynecia* as endemic species can easily become endangered or extinct if their restricted habitat is constantly being changed. Many proposals have arisen in recent years with ideas to "renovate" the Kane Tract. Plans have ranged from constructing mega-malls and housing to hiking trails (Carola 2004). Now, a recent mitigation project is further degrading the road which is currently the only known locality for *C. gynecia* in New Jersey. EarthMark Mitigation Services has started to mitigate 235 of a total 352 acres in the area by constructing 7000 linear feet of berms, submerging a natural gas pipeline and digging up massive stands of the invasive *Phragmites* plant. In turn, they will be replanting native plants such as willow and oak trees and wetland grasses such as *Spartina* sp., bulrush and spike grass (Lamendola 2010). The only access to the Kane Area is the road on which *C. gynecia* inhabits. Dump-trucks, backhoes and bulldozers are constantly trekking and uplifting the soil and water from the puddles found on the road.

Tina Schvejda, executive director of the Meadowlands Conservation Trust, believes that this "enhancement" of the Meadowlands will bring back migratory birds and will once again be considered a flyway stop on their northern journey from South America (Lamendola 2010). It has been shown that clam shrimp are among some of the aquatic

invertebrates that migratory birds, such as Mallards and Northern Pintails, include in their diet so that they can obtain protein, fat and calcium necessary for egg production. The branchiopods require a shorter food-processing time than when the birds eat plants (Krapu and Reinecke 1992, Green and Figuerola 2005). The loss of a single species within an ecosystem can prove to be detrimental for the others that rely on it for food, e.g., migratory birds and clam shrimp. With the intentions of providing public access to an environmental resource, a hiking, walking, biking and birding trail in itself may become a barrier to wildlife if full consideration is not given to the species that inhabit it.

One form of recreation that is both destructive but may also be a potential means of conservation is the use of motorized off-highway vehicles (OHVs) in the Meadowlands. Continuous use of OHVs has many environmental impacts that have been well studied in a wide range of environments all over the world. These include coastal beaches, dunes, and arid landscapes in southwestern USA; grasslands in East Africa; and the arctic tundra (Buckley 2004).

All-terrain vehicles (ATVs) are a type of OHV that frequent the Kane Tract, although it is illegal to operate an ATV in that area (Lamendola 2010). Recreational groups use ATVs for off-road racing and mud-bogging (driving the vehicle through a pit of mud of a set length) (Dennis Francavilla, dirtbiker, personal communication). These practices can be environmentally damaging though there are few studies on their quantitative impacts and direct impacts are often inferred (Hathaway et al. 1996, Buckley 2004).

Environmental impacts of OHVs include: water pollution from fuel and/or oil spills; disruption to waterflow and animal movements; physical action of tires on soils; vegetation and fauna; and the transport of propagules. OHV impact on soil is of particular concern in the Meadowlands which are subject to not only the 250 – 750 lb. all terrain vehicles, but also 30,000 lb dump trucks and plow trucks whose immense tires kick up a lot of dirt and mud while traveling along the road. OHVs apply 5 -15x more pressure on the soil than hiking books and increases during the times when the vehicle is braking, accelerating or skidding. These actions accelerate erosion of the top soil by breaking up and displacing upper soil layers and increase compaction of the deeper soil layers (Hathaway et al. 1996, Buckley 2004).

Despite these environmentally degrading characteristics, ATVs on the Kane Tract are the most probable creator of the puddles in which *C. gynecia* are found. There is also evidence of ATV use in New York and Massachusetts habitats where the clam shrimp are also found (Schmidt and Kiviat 2007, Smith and Gola 2001). In addition, my study supports the hypothesis that OHVs (ATVs and dirt bikes) are dispersers of *C. gynecia* as their larvae were found after rehydration of soil scraped from the treads of an ATV used on the road where the puddles were found.

Although ATVs are the likely vectors of dispersal for this organism, best management practices should include careful management of the vehicle traffic. A balance between the number of passes of an OHV and the degree of erosion and compaction must be determined for the maintenance of the puddle habitats. This is especially important

during the wet season when cysts of these organisms are the most vulnerable. It has been shown that off-highway vehicles can have damaging effects on the cysts of branchiopods. The weight of the vehicles compresses the soil and the forces are great enough to crush and/or deshell the conchostrocan embryos (Hathaway et al. 1996). One recommendation is the restriction of off-road vehicles during the wet season or days of heavy rain.

Human development has accelerated the disappearance of temporary pools at an alarming rate worldwide (Eitam et al. 2004). As we gain a better understanding of *C. gynecia's* habitat, behavior and distribution, this information can be used to create and maintain pools that support the species in the face of pool loss and insure the persistence of *C. gynecia* in its new localities.

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