CONSEQUENCES OF EGG SIZE ON HATCHLING ENERGETICS IN THE DIAMONDBACK TERRAPIN, *MALACLEMYS TERRAPIN*: A GEOGRAPHIC COMPARISON

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by

PHILLIP E. ALLMAN

has been approved for

the Department of Biological Sciences

and the College of Arts and Sciences by

Willem M. Roosenburg

Associate Professor of Biological Sciences

Benjamin M. Ogles

Dean, College of Arts and Sciences

Abstract

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Organisms typically show phenotypic variation across environmental conditions, and in wide-ranging species, this variation is often explained through local adaptation theory. Whether the differences result from evolution or individual plasticity, the variation is often hypothesized to increase the fitness of individuals in that environment. Egg size in oviparous reptiles often varies among populations and can significantly influence key life history traits such as offspring size, growth, sex, survivorship, and ultimately individual fitness. Therefore, understanding the evolution of egg size and clutch size among populations of a single species is central to life history theory. This dissertation examines the energetic consequences of egg size among three populations of the diamondback terrapin, *Malaclemys terrapin*, along a latitudinal gradient. The primary goal was to identify the physiological mechanisms that explain the significance of egg size variation in this long-lived ectotherm.

Specifically, I asked the following questions: 1) Does the quantity or component proportions of energetic lipids in the egg yolk vary among populations, 2) What are the consequences of incubation temperature and egg size on hatchling size, growth,

energetics, and survivorship when raised in different conditions, and 3) What is the rate at which hatchling turtles utilize residual energy stores for six months after hatching.

Female terrapins in northern populations lay relatively small eggs in large clutches whereas, in southern populations, they deposit larger eggs in small clutches. The larger eggs from southern populations have a higher proportion of energetic lipid stores, and under similar incubation conditions, these eggs produce larger hatchings with a higher proportion of residual energy stores. However, these hatchlings also have a higher maintenance metabolism and utilize the energy stores at a faster rate. In warmer climate conditions, the larger turtles grow faster than smaller turtles with less residual yolk. Additionally, these larger turtles have a higher survivorship. Consequently, I suggest females that deposit larger eggs in southern climates are favored through increased survivorship afforded to the offspring, whereas females that deposit small eggs in northern populations are favored through selection on fecundity.

Approved:

Willem M. Roosenburg

Associate Professor of Biological Sciences

Dedication

For Karyn's unending help and support,

I dedicate this dissertation to my wife.

For helping me become the person that I am,

I dedicate this dissertation to my parents:

Richard and Wanda Allman

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Over the past five years, I have been fortunate enough to collaborate with many helpful biologists. Allen Place (Center of Marine Biotechnology, Maryland) welcomed

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Chapter 1: An introduction to the evolution of egg size in oviparous ectotherms

Phil Allman

The Evolution of Life Histories

Darwin (1859) described the evolution of a species through natural selection as a process that results in traits that make populations better adapted for their environment through differential survival and reproductive success. Variation among traits results in some individuals with higher fecundity or survivorship than others, thereby increasing the frequency of the selected trait in the population. Demographic traits that influence reproduction or survival are defined as life history traits and these traits have important implications for the evolution of the species (Roff, 1992; Stearns, 1992). For example, lifetime reproductive success is influenced by such life history traits as age at maturity, size at maturity, and reproductive investment. Life history traits also influence population dynamics through the effects on age at maturity, growth rates and survivorship (Lande, 1993; Denney et al. 2002). Thus, studying life history variation is germane to understanding natural selection in wild populations.

For organisms that are resource limited, adaptive variation in phenotypes can increase resource harvesting, processing or allocation to maximize survivorship and reproduction in that environment (Ricklefs and Miller, 2000). However, because energy allocated to one function cannot be simultaneously allocated to another, a tradeoff exists between functions competing for a similar resource. For example, an increase in the number of offspring produced by an organism may contribute to fitness by increasing fecundity, but it may result in reduced offspring size, future fecundity, or survival of the parent (Stearns, 1992; Schwarzkopf, 1993). Because breeding takes time and resources from other activities, investment in offspring generally diminishes the parent's survival and in many cases, rearing offspring drains the parent's resources such that fewer

offspring are produced later. These tradeoffs are central to life history theory and are often used to explain deviations from unrealized optimal conditions.

Identifying the mechanisms and evolutionary significance of tradeoffs are the main topics of life history studies. The physiological mechanisms that underlie life history tradeoffs can help one identify the functional interactions among the life history traits and the influence of proximate environmental factors. In many cases, these tradeoffs result from the constraint of limited internal resources that cannot be simultaneously allocated to competing demands (Dunham, 1989; Zera and Harshman, 2001). For example, if the internal energy reserves allocated to current reproduction limits the amount of resources available for growth, a physiological tradeoff exists between reproduction and growth (Reznick, 1983). Traditionally, studies of life history tradeoffs have concentrated on how the limited energy is allocated to reproduction, maintenance metabolism, growth, and storage within a single species or across related species (Townsend and Calow, 1981; Congdon et al. 1982).

An organism's life history can be viewed as a set of allocation rules that are based on the organism's interaction with the biophysical, resource, predation, and demographic environments (Dunham et al. 1989). Variation in life history traits is often associated with the influence these environments have on the genetic instructions that produce the phenotype (Wilbur et al. 1974; Ricklefs and Wikelski, 2002). The environment exerts an influence at all life stages, thus creating variation in the phenotype, performance, and ultimately fitness of the organism (Arnold, 1983; Ricklefs and Wikelski, 2002) (Figure 1.1). Natural selection operates as a feeback such that variation in reproductive success among different genotypes results in changes in the genetic composition of the population

(evolutionary change). For example, variation in environmental conditions have been associated with changes in reproductive output (Ricklefs, 1997), growth (Tinkle and Dunham, 1986; Gebhardt-Henrich and Richner, 1998) and survivorship (Aday et al. 2003). However, variation in life history traits may also result directly from plasticity among environmental conditions (Roff, 1992; Stearns, 1992). Whether the differences result from evolutionary changes or plasticity, the variation is assumed to increase the fitness of individuals in that environment (Berven and Gill, 1983).

Maternal Investment and the Evolution of Egg Size

A fundamental life history tradeoff involves the maternal allocation of energy available for reproduction into successful offspring that maximizes the reproductive success of the female (Trivers, 1974). Two evolutionary processes influence the number and size of offspring: 1) selection favoring females that produce the greatest number of offspring (*sensu* Lack, 1947), and 2) selection favoring females that produce larger offspring (Trivers, 1974; Godfray et al. 1991). However, because of limited resources, reproduction requires resources to be allocated among the competing demands of investing in more offspring or investing more to each offspring (Stearns, 1992). By investing equally in the offspring of a single reproductive event, a female potentially maximizes her fitness and those of her offspring (Smith and Fretwell, 1974). Optimal egg size (OES) theory predicts that there is an optimum amount of resources that organisms should invest in each offspring within a given environment (Smith and Fretwell, 1974; Brockelman, 1975; McGinley et al. 1987) (Figure 1.2). However, offspring size often shows considerable variation within species (Garland and Adolph, 1991). Intraspecific variation in offspring size may be due to differences in optimal conditions among

environments or may reflect nutritional or physical constraints on the resources invested to each offspring (Christiansen and Fenchel, 1979; Congdon and Gibbons, 1987). An optimal egg size, in part, is based on the survival advantage given to larger offspring that are produced under the constraint of a finite amount of resources. Therefore, if larger offspring have higher survivorship, then those offspring may offset the cost to the female for producing fewer offspring. However, any factor that changes the relationship between offspring size and probability of survival may influence the optimal egg size. Such factors include predation rate, food availability, and the biophysical environment (Bertram and Strathmann, 1998; George, 1999).

In oviparous reptiles, the egg represents the majority of the female's reproductive investment and larger eggs, typically, represent a larger investment per offspring (Ricklefs and Burger, 1977; Congdon et al. 1983b; Sinervo, 1993; Roosenburg and Dennis, 2005). Minimally, the eggs must contain sufficient energy for development and maintenance of the embryo (PIE), and provide post-hatching energy for maintenance and possibly growth of the hatchling (PIC) before feeding is initiated (Congdon, 1989). For many species, particularly turtles, the residual yolk and hatchling fat reserves constitute post-hatchling parental care and provide the energy for post-hatching dispersal and maintenance.

A major feature of reptile eggs is the high initial lipid content in the yolk (Congdon and Gibbons, 1985). Lipids comprise 27% of the dry yolk mass in the lizard *Morethia boulengeri* (Manolis et al. 1987) and as much as 43% in the snake *Spalerosophis diadema* (Ar et al. 2004). Lipids make up 40.7% of the yolk mass in the turtle *Trachemy scripta* (Caudle, 1984). Yolk lipid is the major energy source for the

developing embryo and provides nutritionally important components for tissue development and growth (Romanoff, 1960). Two lipid types serve these functions and their distinction is imperative to fully understanding lipid use during embryogenesis.

Lipids are heterogeneous substances that are insoluble in water but soluble in organic solvents. They include long-chain hydrocarbons and fatty acids that vary in complexity and size of the long chain bases. Non-polar lipids (neutral lipids) contain one or two fatty acid complexes per molecule and polar lipids contain more than three complexes. Non-polar lipids include triglycerides, cholesterols, fatty acids, and phosphoglycerides. The triglycerides represent the energetic lipid component of the yolk and provide the primary energy source for the developing embryo. The polar lipids represent the structural lipids that are used to build cell membranes during embryogenesis.

The maternal investment to an individual offspring includes the yolk mass that provides the required nutrients to complete development. Depletion of the yolk lipids during embryogenesis will determine how much are available to the individual after hatching. Environmental conditions such as incubation temperature and humidity can influence the rate of lipid consumption, but unfortunately, little information is available on the rate of lipid use after hatching and how that affects hatchling fitness.

Egg Size Variation in Ectotherms

Selection on offspring size varies across time and space and may result in a significant amount of egg size variation among populations of a single species (Mousseau and Roff, 1987; Fox and Czesak, 2000; Messina and Fox, 2001). Much of this variation is associated with differences in female body size, but differences remain after

accounting for maternal body size. For example, the lizard *Anolis carolinensis* in central Florida has a mean egg mass of 0.15 g whereas a population in Tennessee has a mean egg mass of 0.28 g (Michaud and Echternacht, 1995). Although adult females are larger in Tennessee, regression analysis clearly indicates the eggs are disproportionately larger than would be expected from allometry alone. In a widespread iguanid lizard, *Sceloporus occidentalis*, egg size decreases with latitude and altitude (Sinervo, 1990). By experimentally manipulating egg size, Sinervo (1990) was able to demonstrate that many traits associated with fitness such as sprint speed are affected by egg size, but that other traits, such as growth, reflect genetic differentiation among populations. Sinervo and McEdward, (1988) suggested that evolutionary changes in maternal investment per offspring influences offspring fitness traits that are related to size. To understand the causal relationships among egg size, offspring size, offspring fitness and how they may vary across different environments, we should employ a comparative approach that evaluates the underlying physiological constraints and energetic needs among populations. Turtles provide an excellent system to address the evolution of egg size and the question of optimization to local environments.

Many species of turtles exhibit decreasing egg size with increasing latitude. Female *Trachemys scripta* in Panama lay ellipsoidal eggs of 42 X 29 mm with a mean mass of 20.6 g (Moll and Legler, 1971), whereas females in Louisiana deposit eggs of 38 X 23 mm in size and 17.1 g in mass (Cagle, 1950). Additionally, individuals from a population in Illinois deposit 6.1 X 15.4 mm eggs with an average mass of 9.7 g (Cagle, 1944). Iverson et al. (1997) reported that *Chelydra serpentina* egg size decreases with latitude, but this effect is absent when corrected for female size. Seigel (1980) suggested

a cline in *Malaclemys terrapin* egg size from 7.7 g in New Jersey to 12.5 g in Florida. Most hypotheses that attempt to explain latitudinal egg size variation focuses on how environmental conditions may alter the relationship between hatchling size and survivorship, thus creating variation in the expected optimal egg size (Figure 1.2).

One such hypothesis poses that in temperate regions productivity is higher in the spring months than in the tropics, so reproduction would be less resource-limited and females can convert more energy into more, or larger, young during spring (Cody, 1966; MacArthur, 1972). This hypothesis fits the latitudinal trend in some turtles (Iverson et al. 1993), but many turtles only found in temperate regions possess this trend. This hypothesis does not explain why resources wouldn't be directed into increasing egg size and investment per offspring instead of increasing the number of offspring.

Another hypothesis predicts that decreased predation risk at cooler temperatures (Pianka, 1988) will favor females that produce more hatchlings since the reduced predation pressure eliminates the advantage of producing larger offspring. This assumes that small eggs have the same long-term fitness as larger eggs. Iverson and Smith (1993) indicated that size-selective predation on turtle hatchlings is rare in the painted turtle (*Chrysemys picta*), therefore this hypothesis does not explain clines in egg size variation.

A final hypothesis relates egg size to the length of incubation and the duration of suitable environmental conditions for embryonic development. The primary prediction is that shorter summers at higher latitudes require embryos to develop faster (Iverson et al. 1993). Ewert (1979) demonstrated that incubation times for turtle eggs are shorter at high latitudes and at least one study has shown small turtle eggs hatch sooner than larger eggs of a particular species (Packard et al. 1989). Conversely, large eggs are favored at

high latitudes in the frog *Rana temporaria* because they produce faster growing tadpoles that can metamorphose sooner (Cummins, 1986). Although there is some support for this hypothesis, other factors such as nest site choice need to be accounted for in the correlation of egg size and incubation time.

Explaining egg size variation in ectotherms has been a target of both empirical and theoretical studies attempting to understand the tradeoff of egg size and clutch size within the framework a local adaptation hypothesis. Unfortunately, only a few studies have attempted to identify the physiological or demographic mechanisms behind such intraspecific variation in egg size.

Chapter Summaries

Oviparous reptiles deposit energy-rich eggs, with varying amounts of albumen, yolk, and other components, into the terrestrial environment where embryonic development is completed. Thus, eggs are deposited in unpredictable environments where the female has minimal post-ovipositional control over the conditions in which the embryos will develop. Conditions such as temperature and humidity influence the embryo's water budget, energy utilization, incubation duration, hatchling mass, and hatchling survivorship (Booth, 1998; Rhen and Lang, 1999; Congdon, 1990). Therefore, hatchlings from diverse environments may have different energy requirements and require different amount of resources from the yolk to survive. If so, egg size may be under strong selection that may lead to local adaptation that optimizes the hatchling resource demand to maximize offspring size, growth, and/or survivorship in the exposed environment.

The diamondback terrapin, *Malaclemys terrapin*, is a good model system for studying egg size variation in long-lived ectotherms. This species has a broad geographic range, occurring along the eastern and southern coasts of the United States, and only inhabits the coastal estuaries and saltwater marshes. There is substantial variation in the abiotic environment throughout their range, however because they are coastal, there are no confounding climate factors associated with altitude or continental patterns. Individual turtles show high site fidelity and increased genetic differentiation with geographical distance (Hauswaldt and Glenn, 2005). Additionally, this species exhibits considerable variation in life history traits among populations. For example, females in southern populations have a smaller adult body size than females in northern populations (Montevecchi and Burger, 1975). Egg size is uniform within a clutch (Roosenburg and Dennis, 2005), but varies among clutches and populations (Seigel, 1980). Females will deposit multiple clutches of eggs throughout a nesting season. For many populations, females are known to nest as many as three times within a single season (Seigel, 1980; Roosenburg, 1994; Butler, 2000). The terrapin nesting season varies with latitude such that nesting season is shifted earlier in the year in southern populations and is later in the year for northern populations (Seigel, 1980; Butler, 2000; Feinburg and Burke, 2003).

In this dissertation, I present differences in egg size and clutch size from three populations of diamondback terrapins and describe the variation in non-polar lipids (NPL) and lipid components from those populations (Chapter 2). Next, I explore the consequences of variation in yolk lipid has on hatchling energetics (Chapters 3 & 5), growth (Chapter 4), and survivorship (Chapter 4) among eggs incubated at different temperatures.

First, I describe variation in egg mass, length, width, and volume from eggs collected in Rhode Island (RI), Maryland (MD), and South Carolina (SC) during the beginning of the nesting season for each site (Chapter 2). Egg size and clutch size differed among populations indicating females produce larger clutches with smaller eggs at high latitudes and small clutches of larger eggs at lower latitudes. Lipid analysis also indicated that eggs from SC contained yolks with a higher proportion of NPLs than eggs from MD or RI. Therefore, female terrapins in SC are depositing larger eggs that contain a larger energy source to the developing embryo. The major component of the yolk lipids was triacylglycerol (energetic lipid) for all populations. However the proportion of triacylglycerol varied with population, demonstrating the necessity of quantifying lipid components when asking energetic questions. These data indicate a strong shift in reproductive strategy where females in northern populations invest in higher fecundity and females in southern populations, through increased egg size and energy reserves, invest in larger offspring.

Because environmental conditions, such as incubation temperature, can influence patterns of hatchling development, the remaining chapters explore the consequences of egg size and environmental conditions on hatchling energetics and fitness traits. In Chapter 3, I examine the consequences of egg size and incubation temperature on hatchling size, energy reserves, and sex among eggs collected from RI, MD, and SC. For all populations, incubation period decreased with incubation temperature, however an interaction effect indicates the influence is population specific. Hatchling mass decreased and NPL mass increased with incubation temperature for all populations. The smaller eggs from RI produced hatchlings of smaller size and with less energy stores at all

incubation temperatures than eggs from MD or SC. My data indicate larger eggs produce larger hatchlings that contain more energy reserves that may be used for maintenance metabolism or growth. Substrate temperatures measured in the field suggest eggs in southern populations are incubating at higher temperatures that will produce a relatively smaller hatchling with higher energy stores. If the available energy reserve provides a fitness advantage to the offspring, then it is likely that the energetic demand of the hatchling may explain latitudinal variation in egg size (energetic constraint hypothesis).

To examine if egg size variation can be at least partially explained through the environmental influence of hatchling energetics, I conducted a common-garden experiment where hatchlings from RI, MD, and SC incubated at 27° C, 28.5° C, or 30° C were randomly assigned to growth rooms that mimicked the water temperature and light cycle of RI (cold room), MD (warm room), or SC (hot room) (Chapter 4). For all incubation temperatures, hatchlings from SC had a higher mass-specific metabolic rate, indicating neonates from SC have a higher maintenance energetic demand. Additionally, the RMR increased with incubation temperatures, suggesting that warmer incubation temperatures in SC will further increase the neonate's energetic demands. All hatchlings in the hot growth room grew to a larger size than the warm or cold growth room; however, since I mimicked the actual temperatures, hatchling mass decreased throughout the study in a population-specific pattern. Hatchlings from Rhode Island in the hot room lost the most mass during the six month study. Interestingly, after three months of growth in the warm and cold room, population source or hatchling size did not influence the rate of mass change each month. Furthermore, survivorship was high throughout the

study, however survivorship in hatchlings from Rhode Island decreased after four months in the hot room.

In chapter five I examine the influence of environmental conditions on the rate at which turtles utilize residual energy stores for six months after hatching. For each population, hatchlings raised in the hot treatment room utilized residual energy stores at a faster rate than hatchlings in the cold treatment room. Additionally, hatchlings from SC eggs maintained a higher amount of energy stores than hatchlings from RI or MD. The pattern of growth among treatment groups suggest some of the energy stores are being allocated toward growth as well as maintenance metabolism.

In summary, I have demonstrated that incubation conditions and post-hatching environmental conditions influence the energetics of embryonic and hatchling turtles. Populations in southern climates are exposed to warmer conditions than populations in northern climates. This warmer climate results in hatchlings with a higher metabolism and rate of energy utilization. In this climate, larger eggs that produce larger hatchlings with more energy stores are favored through increased survivorship over smaller hatchlings. In northern climate conditions, larger hatchlings do not have a growth or survivorship advantage over smaller hatchlings. Therefore, these data support the energetic constraint hypothesis which states that females that deposit large eggs in southern climates are favored through increased offspring fitness, whereas females that deposit small eggs in northern climates are favored through increased fecundity.

Chapter 2: Geographic variation in egg size and composition in the wide-ranging diamondback terrapin, *Malaclemys terrapin*

Phil Allman, Allen Place, Willem Roosenburg

Introduction

Oviparous reptiles deposit eggs that must provide sufficient energy to cover the metabolic cost of producing a fully developed hatchling, and in turtles, the egg includes significant post-hatching energy stores. The yolk contains most of the material utilized by the developing embryo although water, oxygen, and a few inorganic ions are required from outside of the egg (Thompson, 1989). Protein and lipid material (1:2 ratio) make up over 90% of the yolk's dry mass (Noble, 1991; Thompson et al. 2001). The remaining dry yolk materials are composed of inorganic ions, water-soluble vitamins, and carbohydrates (Speake and Thompson, 1999). Triacylglycerols, phospholipids, cholesterols, and cholesteryl esters are the major lipid classes in reptile yolks. Triacylglycerol comprises from 70% to 87% of the total lipids in reptile eggs (Ballinger et al. 1992; Nagle et al. 1998) and is the primary energy source for the developing embryo (Derickson, 1974; Congdon and Tinkle, 1982).

With the exception of the energy used for choosing a nest site, chelonian eggs contain all the energy allocated by the female to each offspring. The egg provides energy for parental investment in embryogenesis (PIE) and energy for parental investment in care (PIC) (Congdon, 1989). Residual yolk and hatchling fat bodies constitute PIC that is used for post-hatching maintenance and dispersal. Also, because most turtles hatch at the end of the resource-rich growing season, PIC may provide enough energy to survive through the period of negative energy balance (Nagle et al. 1998) to the upcoming spring. The egg represents the majority of the female's reproductive investment and larger eggs, typically, represent a larger maternal investment per offspring (Ricklefs and Burger, 1977; Congdon et al. 1983b; Roosenburg and Dennis, 2005). When incubation

conditions, such as increased temperatures, require more energy to complete embryogenesis, less energy reserves are available for the hatchling (Congdon and Gibbons, 1990; Thompson et al. 1999; Lance and Morafka, 2001).

In many oviparous reptiles, egg size influences offspring fitness traits such as hatchling size (Sinervo and Huey, 1990; Roosenburg and Kelley, 1996), swimming speed (Miller et al. 1987; Miller, 1993), and running speed (Sinervo and Huey, 1990). Janzen et al. (2000) provided evidence that larger red-eared slider (*Trachemys scripta*) offspring have higher survivorship during post-emergence migration, suggesting females may increase offspring fitness by producing larger offspring. The benefit of larger offspring size may be improved locomotor performance that enhances the ability to escape predators. However, at least one long-term experiment indicated hatchling survival was not correlated with body size in the snapping turtle, *Chelydra serpentina* (Congdon et al. 1999). Thus, there may be situations where there is no benefit to larger offspring size (Ferguson and Fox, 1984; Laurie and Brown, 1990). For example, when predation and competition pressures are reduced, the benefits provided larger individuals of the lizard *Uta stansburiana* are lost (Fox, 1983)

An alternative mechanism for increasing offspring fitness is to increase the amount of energy reserves provided to the hatchling (Congdon, 1989, Nagle et al. 1998). Bobyn and Brooks (1994) showed intermediate sized snapping turtle hatchlings that had greater yolk reserves grew faster and survived longer in captivity. Experimental yolk reductions have resulted in reduced yolk sack in birds (Finkler et al. 1998) and smaller lizard hatchlings (Sinervo and Huey, 1990). Additionally, in a comparison of 12 turtle species, Congdon and Gibbons (1985) suggested turtle species that overwinter in the nest

cavity allocate proportionately more lipid to the eggs than do species that emerge in the fall, however Nagle et al. (1998) did not see this pattern in kinosternid turtles.

The diamondback terrapin, *Malaclemys terrapin*, is a wide-ranging emydid turtle that has a semi-continuous range from the northeast United States coast to the Texas coast. The terrapin exhibits geographic variation in reproductive traits (Seigal, 1984; Roosenburg, 1994) that may be a result of local adaptation to environmental conditions. Terrapin egg size varies among clutches within a single population but varies little within a clutch (Roosenburg and Dunham, 1997). In the present study, I examined egg size variation from three populations along a latitudinal gradient. I also characterize the relationships between egg wet mass, dry mass, water content, non-polar lipids (NPL) (energetic lipids), and lean dry mass across different populations and throughout a complete nesting season. Water content, non-polar lipids, and lean dry mass are known to increase with egg size in a single population (Roosenburg and Dennis, 2005), however these components have not been compared among populations or throughout the nesting season. Finally, I also show the lipid class proportions of eggs from different populations to determine if females allocate a different proportion of storage lipid components in different populations.

Materials and Methods

I located terrapin nests during the 2004 nesting season on Grice Island, South Carolina (32°47'N, 79°56'W), Patuxent River, Maryland (38°27'N, 76°39'W), and Nokum Hill, Rhode Island (41°45N, 71°31'W). Nests that were less than 12 hours old, determined by fresh tracks and the presence of unchalked eggs, were excavated to count, measure (length and width to the nearest mm), and weigh (to the nearest 0.1 g) each egg.

I calculated egg volume using the ellipsoid formula [volume = (π/6) (length) (width^2)] (Iverson and Ewert, 1991). From MD and RI, I randomly froze (-20° C) two eggs from a subset of clutches for lipid analysis. In SC, due to small clutch sizes, only one egg from a subset of clutches was collected and frozen (-20° C). Furthermore, because it was difficult to locate fresh nests in South Carolina, I captured gravid females and induced oviposition using oxytocin injections within 48 hours of capture (Ewert and Legler, 1978). All eggs were collected at the beginning of nesting season (RI: June 20, MD: June 1, SC: May 20) and in less than a two week period for each population, thus a different female produced each clutch and the nests most likely represent the first clutch of the season for that female.

I also collected three eggs from freshly deposited nests during the beginning (June 1), middle (June 25), and end (July 20) of the 2003 nesting season on the Patuxent River, Maryland. Diamondback terrapins deposit two or three nests in a single season. Collecting eggs throughout the nesting season in 2003 allows us to look at the variation in egg size and energy content among clutches throughout the nesting season. Previous work illustrates that NPL mass does not vary within a clutch (Roosenburg and Dennis, 2005) so I am confident the collected eggs are representative of the entire clutch.

Frozen eggs were transported to Ohio University and thawed before the yolk was separated from the shell and albumen. The components were dried separately at 60° C to a constant mass (to the nearest 0.0001 g). I ground the dried yolk to an even consistency using a mortar and pestle. The sample was transferred to a new pre-weighed Whatman cellulose thimble (33 X 80 mm) and then placed into a SoxTech HT2 1045 extraction unit. Petroleum ether was refluxed through the samples for 90 minutes at 100° C, the

samples were then rinsed for 45 minutes, and the remaining solvent was evaporated for an additional 15 minutes. I then dried the extracted lipids in a drying oven at 60° C for 60 minutes before weighing to the nearest 0.0001 g. The calculated sample recoveries after transferring to the extraction thimbles ranged from 96% to 99%. Previous work indicates this method results in the extraction of 100% of the non-polar lipids within the sample (Roosenburg and Dennis, 2005). I extracted six empty extraction thimbles to serve as a blank control. The lean dry yolk mass was calculated as the dry yolk mass – lipid dry mass.

I used thin-layer chromatography (TLC) and flame-ionization detection (FID) to quantify the yolk lipid components (Center of Marine Biotechnology, Baltimore, Maryland). A 0.1 g sub-sample of yolk was dissolved in 4 ml of 2:1 ratio methylene chloride – methanol to extract the total lipids. I homogenized the material for three minutes and then centrifuged the sample at 2000 rpm for 15 minutes. This procedure was repeated to ensure high extraction efficiency (89% - 94%). The extracted lipids were washed with 0.88% KCl in water and then allowed to dry overnight.

I reconstituted the dry lipids in methylene chloride at a concentration of 10 µg / µl. After solvent focusing (Ackman et al. 1990), samples were spotted on Chromarod silica-gel coated glass rods in triplicates. The samples were focused in a 1:1 ratio of methanol – chloroform and then developed for 45 minutes in an 85:15:0.1 ratio of hexane – diethyl ether – formic acid to separate sterol esters, triacylglycerols, and cholesterol. Then, I scanned the rods in an Iatroscan TH-10 TLC/FID Analyzer using a hydrogen flame with a gas flow rate of 150 ml / min. Each rod was scanned for 30 seconds to produce the component lipid peaks. I ran a series of standards, in triplicate, for esters,

triacylglycerols, and cholesterol to produce standard curves for analysis ($r^2 > 0.98$ for all curves). The final component values for each sample were calculated as the mean of each triplicate set. Component values obtained from the Iatroscan were also multiplied by total wet yolk mass to determine the total content value for each yolk.

All data were analyzed using SAS for PC (version 9.1.3 SAS Institute, Cary, NC). Egg size and lipid composition data were log-transformed, as appropriate, to meet the assumption of normality for all parametric analyses. I used a MANOVA to test for differences in egg size and then used a nested ANOVA to partition the variation of each egg measurement among location (fixed effect) and clutch (nested effect). Linear regression analysis was used to determine the relationship among egg size and lipid composition. After revealing a strong clutch effect, egg and lipid data were analyzed using a full model ANCOVA with clutch mean values as treatment effects and lean dry yolk mass as the covariate. I also tested for homogeneity of slopes by testing for a significant population by covariate interaction.

Results

Egg size (MANOVA, Wilk's λ=0.55, $F_{6,920}$=53.1, *P=<0.001*) and clutch size (ANOVA, $F_{2,40}$=119, *P*<0.0001) differed among populations indicating large clutches with small eggs occures at high latitudes and small clutches with larger eggs at low latitudes. Mean egg mass increased from 8.4 g (Rhode Island) to 10.4 g (South Carolina) and mean clutch size decreased from 16.1 eggs (Rhode Island) to 6.0 eggs (South Carolina) with decreasing latitude (Table 2.1). Egg mass ($F_{2,423}$=13.4, *P*<0.0001), egg length ($F_{2,423}$=18.1, *P*<0.0001), and egg volume ($F_{2,423}$=8.3, *P*=0.0001) differed among populations. There was also a clutch effect on egg mass (ANOVA,$F_{40,423}$=55.5,

$P<0.0001$), egg length (ANOVA,$F_{40,423}=26.7$, $P<0.0001$), and egg volume (ANOVA,$F_{40,423}=44.1$, $P<0.0001$). Although egg width differed significantly among clutches (ANOVA,$F_{40,423}=41.1$, $P<0.0001$), egg width did not vary among populations (ANOVA,$F_{2,423}=3.0$, $P=0.06$). Because of the strong clutch effects, all further analyses were conducted using clutch mean values as independent measurements. Additionally, egg size in Maryland (Egg Mass: $F_{2,25}=1.9$, $P=0.1886$, Egg Length: $F_{2,25}=0.12$, $P<0.8867$) did not vary among eggs collected at the beginning, middle, and end of nesting season.

Changes in egg size were accompanied by both changes in egg length and width in all populations. Clutch mean egg length (RI: $\rho=0.94$, $P<0.0001$, MD: $\rho=0.53$, $P=0.04$, SC: $\rho=0.46$, $P=0.05$) and clutch mean egg width (RI: $\rho=0.97$, $P<0.0001$, MD: $\rho=0.90$, $P<0.0001$, SC: $\rho=0.90$, $P<0.0001$) were correlated with clutch mean egg mass among all populations. The slopes of the fit lines for mean egg length ($F_{2, 37}= 0.95$, $P=0.3943$) and mean egg width ($F_{2, 37}= 1.47$, $P=0.2423$) do not vary among populations (Figure 2.1); suggesting the relationship in egg size and mass is similar for all populations.

Egg energy content decreased with latitude, indicating eggs in the south contained greater energy stores than eggs in the north. The mean wet yolk mass increased from 3.95 g (RI) to 4.6 g (SC) (ANCOVA,$F_{2,32}=4.5$, $P=0.0182$) (Table 2.2). There was a significant difference in NPL mass per unit lean dry mass among the three populations (ANCOVA,$F_{2,32}=12.3$, $P=0.001$). The NPL mass increased from 0.384 g (RI) to 0.668 g (SC), and contributed from 22.3% (RI) to 31.8% (SC) of the dry yolk mass. Additionally, water (ANCOVA,$F_{2,9}=1.5$, $P=0.2727$) and NPL (ANCOVA,$F_{2,9}=0.6$, $P=0.5648$) content was similar in MD eggs collected through the nesting season. For all populations, the NPL mass was positively correlated with yolk lean dry mass (RI:

ρ=0.86, *P*=0.0003, MD: ρ=0.74, *P*=0.022, ρ=0.66, *P*<0.0077) (Figure 2.2). The amount of water per unit lean dry mass varied among populations ($F_{2,32}$=3.4, *P*=0.047), and ranged from 2.19 g (Rhode Island) to 2.42 g (SC). Egg yolk from South Carolina and Maryland had similar mean lean mass (1.5 g), but was significantly larger than lean mass in Rhode Island eggs (1.37 g) ($F_{2,32}$=3.5, *P*=0.042). The slopes of the relationship of lean dry mass with water mass ($F_{2,30}$=0.35, *P*=0.7095) and lean dry mass with NPL mass ($F_{2,30}$=0.43, *P*=0.6522) were similar for all three populations.

The major component of the yolk lipids was triacylglycerol (energetic lipids) for all populations. The total triacylglycerol in eggs increased from 0.373 g (RI) to 0.654 (SC) with decreasing latitude (ANCOVA,$F_{2,19}$=31.2, *P*<0.0001), but the proportion of triacylglycerol to total extracted lipid actually decreased from 88% (RI) to 82% (SC) (Table 2.3). Cholesterols and phospholipids (both structural lipids) made up a smaller percentage of the total yolk dry mass for all populations but increased in mass with decreasing latitude (Cholesterol: $F_{2,19}$=5.12, *P*=0.0167; Phospholipid: $F_{2,19}$=14.93, *P*<0.0001).

Triacylglycerol (RI: ρ=0.78, *P*=0.0214, MD: ρ=0.92, *P*=0.0036, SC: ρ=0.64, *P*=0.0404) and cholesterol (RI: ρ=0.41, *P*=0.0310, MD: ρ=0.92, *P*=0.0039, SC: ρ=0.73, *P*=0.0379) were significantly correlated with lean dry yolk mass among all populations (Figure 2.3). Pospholipid mass was correlated with lean yolk mass only in Maryland (RI: ρ=0.01, *P*=0.9951, MD: ρ=0.96, *P*=0.0007, SC: ρ=0.09, *P*=0.8389). The slopes of the fit lines for the relationship of triacylglycerol and cholesterol with lean yolk mass did not differ among populations (Triacylglycerol: $F_{2,17}$=0.075, *P*=0.9276; Cholesterol: $F_{2,17}$=

2.06, P=0.1582), but the relationship between phospholipids and lean yolk mass did vary with population ($F_{2,17}$=3.685, P=0.0449).

Discussion

This study suggests a geographic cline in egg size, such that egg mass and length increases with decreasing latitude among the three populations of *Malaclemys terrapin*. The associated decrease in clutch size suggest a tradeoff, with fewer eggs being deposited during a single reproductive event in southern populations. Interestingly, clutch size and clutch mass in Rhode Island is more than twice the amount in South Carolina. Larger eggs within, and among, populations contain more energetic lipids than smaller eggs although the eggs in Rhode Island contained a higher percentage of triacylglycerol (88%) than in other populations. This indicates a shift in reproductive strategy where females in northern populations invest in larger clutches of small eggs and females in southern populations, through increased egg size and more energy reserves, invest in larger offspring and greater maternal care for the offspring.

I found no difference in egg mass, egg length, or egg width among eggs collected through a single nesting season in Maryland. Additionally, there were no differences in lipid content among the eggs, suggesting that on average, female investment to reproduction is similar throughout the nesting season. Follicle enlargement typically begins in the fall (Ernst, 1971; Congdon and Tinkle, 1982) with final follicle maturation in the spring (Congdon and Tinkle, 1982), but for species such as *Chrysemys picta*, *Trachemys scripta*, and *M. terrapin* that deposit two or more clutches in a season, follicles for several clutches typically develop simultaneously (Congdon and Gibbons, 1990). Unlike our findings, second clutches in *T. scripta* have proportionally more

energy than first clutches, but this extra energy is most likely from harvested energy and not stored reserves (Congdon and Gibbons, 1990).

Similar to previous studies with this species (Roosenburg and Dennis, 2005), egg mass was a stronger correlate with egg width than with egg length. But, there were no differences in mean egg width among the populations suggesting that within population variation in egg mass is influenced by egg length and width but among population variation in egg mass is influenced primarily by egg length. These different patterns may reflect a constraint in egg morphology due to female adult size. Although female body size was not measured in this study, female terrapins in southern populations are generally smaller than females in northern populations (Montevecchi and Burger, 1975). To enlarge egg size, females in southern populations may have to increase egg length more than width due to a relatively smaller pelvic aperture size in these populations (Tucker et al. 1978; Congdon and Gibbons, 1987, Clark et al. 2001). However, within a given population, egg size may increase through increased length and width if there is a strong correlation in egg size and female body size. Such a correlation has been documented in other species of turtles (Congdon et al. 1987; Iverson and Moler, 1997; Bowden et al. 2004), but further study is needed to resolve this issue.

The positive relationship among NPL mass and yolk lean mass was not different among populations so a change of lean mass is associated with a proportionally similar change in NPL mass. However, total NPL mass and water content were larger in eggs from South Carolina and the proportion of non-polar lipids to dry yolk mass (14.8%) and dry yolk mass (31.8%) was higher than proportions in eggs from Maryland or Rhode Island. Females in southern populations are not only building larger eggs, but the

increased parental investment is also a function of providing the egg with more non-polar lipids that represent a higher proportion of the yolk mass. Rowe (1994) described egg size variation in populations of *C. picta*, but did not see differences in the proportion of non-polar lipids (Rowe et al. 1995).

Triacylglycerol is the primary component of egg lipids in all three populations (82-88%). These values are comparable to *Kinosternon* bauri (86%), *K. subrubrum* (85%), *S. odoratus* (84%), and *Apalone mutica* (82%) (Nagle et al. 1998; Nagle et al. 2003), but are higher than those reported for *Chelydra serpentina* (68%), *Chrysemys picta* (72%), and *Emydoidea blandingii* (70%) (Rowe et al. 1995). Interestingly, there was a general increase in the proportion of triacylglycerol to total lipids with increasing latitude. The total lipids in RI contained a higher proportion of triacylglycerol (88%) than in lipids from SC eggs. However, the SC eggs contained a larger amount of total triacylglycerol in the egg. This change is associated with a decline in the percentage of phospholipids allocated to the total lipids. Phospholipids function primarily as structural components of cell membranes so this tradeoff in providing a higher proportion of triacylglycerol at the expense of phospholipids may influence the relationship between offspring size and egg size among populations.

Cholesterol is a structural lipid that is often extracted in non-polar solvents and may influence the accuracy of using NPL measurements as an index of available energy stores (Nagle et al. 1998). This study found that cholesterol is a minor component of the total lipids (1.5%) and that the proportion does not vary among populations. The variation in the energetic NPL (triacylglycerol) was complemented by differences in

quantities of polar lipids, suggesting that NPL measurements alone should not be used as an accurate measure of the available energy stores in eggs of this species.

Parental investment to an egg includes the allocation of energetic lipids that are used for embryogenesis (PIE) and then as energy reserves to be used by the hatchling after emerging from the egg (PIC) (Congdon, 1989). Selection acting on the amount of energy allocated to an egg will be influenced by the incubation environment and the hatchling environment (Fischer et al. 1991) when energy resources influence offspring fitness. Previous reports indicate turtle species with hatchlings that overwinter in the nest chamber are provided with a higher amount of energy reserves (Congdon et al. 1983b; Rowe et al. 1995), but Nagle et al. (1998) reported that although differences in egg lipids existed among species, hatchling lipids were not different. This pattern may result from different energy utilization patterns during embryogenesis as a result of extended incubation periods (Ewert, 1985). Species with extended incubation periods may require a larger energetic investment to complete embryogenesis, thus eggs from these species will have a higher proportion of energetic lipids in the eggs, but not necessarily the hatchlings (Nagle et al. 1998).

This study allowed me to examine energy allocated to eggs of a single species in populations that overwinter in the nest chamber (Rhode Island) and populations that exhibit immediate emergence (South Carolina). Given that female turtles in Rhode Island are allocating a smaller proportion of non-polar lipids to the eggs than females in South Carolina, this species demonstrates a pattern of increasing energy allocation in populations with immediate emergence. During winter months, the northern populations are exposed to cooler temperatures and shorter days that result in hibernation (Yearicks et

al. 1981) and most likely reduced metabolic rates and energy expenditures (Reese et al. 2002). Therefore, it is likely that hatchlings from southern populations that emerge immediately after hatching and that do not hibernate will have higher post-hatching energy expenditure than hatchlings from northern populations. This variation in post-hatching energy demand may explain the egg size and lipid variation described in this study.

Additionally, variation in the incubation environment among populations may influence the amount of energy used during embryogenesis (Rhen and Lang, 1999). Incubation temperature and soil moisture can influence the rate of development and possibly the rate of energy utilization during embryogenesis (Gutzke et al. 1987; Janzen et al. 1990). Just as hatchling energy demand may influence egg size among populations, if warmer incubation temperatures in South Carolina require more energy to complete an embryo, then selection may be acting to increase allocation of energy to the yolk for embryogenesis, thus increasing egg size.

The population variation described here indicates egg size and yolk energy allocation may be tightly coupled to the incubation and hatchling environment through the energetic demand of the developing embryo and hatchlings. This may lead to local adaptation patterns that optimize maternal investment in offspring. Comparisons of embryological energetics and hatchling energy utilization, growth, and survivorship need to be described among different populations to further test this hypothesis.

Chapter 3: Variation in maternal investment to offspring size: population- and environment-specific effects on hatchling phenotype

Phil Allman and Willem Roosenburg

Introduction

Variation in offspring size is widely reported across vertebrate taxa and has been the focus of considerable empirical and theoretical work (fish: Fleming and Gross, 1990; amphibians: Cummins, 1986; turtles: Iverson et al. 1993; birds: Horak et al. 1995). In most cases offspring size tends to decrease with increasing latitude (Iverson et al. 1993; Encabo et al. 2002; Allman, Chapter 2), although many of these species follow Bergman's rule and exhibit larger adult body sizes at higher latitudes (Quin et al. 1996; Ashton and Feldman, 2003; Meiri and Tamar, 2003). If offspring size has fitness consequences, the observed differences in offspring size among populations may be indicators of differences in selection pressure for this trait. In this paper, I develop the "energetic constraint hypothesis" to provide a mechanistic explanation for egg size differences exhibited among populations wide-ranging oviparous reptile, the diamondback terrapin, *Malaclemys terrapin* (Allman, Chapter 2). This hypothesis asserts that females in southern populations deposit larger eggs than females in northern populations because hatchlings in southern climates have a higher energetic demand and require larger energy stores to survive through the period of a negative energy budget into the following spring.

Strategies of reproductive allocation into offspring size and number are commonly interpreted through optimality models that predict offspring size is optimized such that the increase in maternal fitness from producing larger offspring (with higher survivorship) is balanced with the decrease in fitness from reducing the number of offspring (Smith and Fretwell, 1974; Brockelman, 1975). This argument assumes that reproductive output is constrained by maternal reproductive capacity or resources,

resulting in a tradeoff among number and size of offspring. Additionally, optimal egg size theory assumes that offspring survivorship increases with size. Indeed, larger offspring of many species exhibit greater fitness than smaller offspring (Bagenal, 1969; Einum and Fleming, 2000; Janzen et al. 2000; Marshall et al. 2003), however, offspring size is not always maximized through maternal investment (Einum and Fleming, 2000). In some turtles, large females deposit eggs that are smaller than the morphologically constrained limit (Clark et al. 2001) or the size expected to maximize hatching success or neonatal survivorship (Gutzke and Packard, 1985; Janzen et al. 2000).

In addition, fitness components such as growth and survivorship are not always correlated with offspring size (Wicklund and Karlsson, 1984; Kaplan, 1992; Ruohomäki et al. 1993), suggesting that the benefits of a large size are reduced in some environments (Berven and Chadra, 1988). For example, Einum and Fleming (1999) found that fitness measures in juvenile brown trout were not associated with body size in laboratory conditions with reduced competition; but under field conditions with increased competition, individuals from larger eggs had growth and survival advantages over individuals from smaller eggs. Given the interaction between the environment and offspring fitness traits, it is reasonable to expect optimal offspring size will vary among environments. The effects of environmental variation on optimal offspring size have been widely described (McGinley et al. 1987; Mousseau and Fox, 1988), and have been used to explain intraspecific variation in life history traits (Bernardo, 1996; Benton et al. 2005; Räsänen et al. 2005)

In oviparous reptiles, female investment in offspring can be divided into two components: investment in embryogenesis (PIE) provides energy for the successful

development of a complete hatchling, and female investment in care (PIC) provides additional energy for post-hatching growth and survivorship (Congdon, 1989). The PIC is represented by energetic stores in the yolk and fat bodies that remain available to the hatchling immediately following emergence from the egg. This component of parental investment is utilized during the early life stages prior to feeding and attaining a positive energy balance (Congdon, 1989; Nagle et al. 2003). Egg size reflects the amount of female investment and larger eggs, typically, represent a larger maternal investment (Ricklefs and Burger, 1977; Congdon et al. 1983a; Roosenburg and Dennis, 2005). Parental investment in care often represents up to 60% of the original egg lipid (Nagle et al. 1998), suggesting this energy store represents a significant portion of the energy invested to individual offspring. However, incubation conditions such as temperature (Allsteadt and Lang, 1995; Rhen and Lang, 1999a; Lin et al. 2005) and humidity (Packard et al. 1983) influence the amount of energy required to complete development, and therefore, determine the amount of energy remaining to the hatchling after emerging from the egg (Congdon and Tinkle, 1982; Congdon and Gibbons, 1990; Rowe et al. 1995). For example, low incubation temperatures reduce embryonic metabolic rate, thus increasing incubation time, embryonic energy utilization, and hatchling size (Christian et al. 1986; Booth, 1998; Booth and Astill, 2001). Thus, incubation temperature can be a strong modulator of maternal investment.

In this paper, I identify the energetic constraint hypothesis as an explanation for egg size variation in the diamondback terrapin, *Malaclemys terrapin*. This species is limited to coastal waters in the eastern and southern United States, and ranges almost 20 degrees of latitude (Ernst et al. 1994). Adult females in northern populations are larger

than adult females in southern populations (Montevecchi and Burger, 1975), however the larger females deposit a larger amount of smaller eggs than females in southern populations (Allman, Chapter 2). The bigger eggs contain a proportionally larger amount of non-polar lipids, indicating that females in southern populations are providing the developing embryo with a disproportional higher amount of energy (Allman, Chapter 2). In the present study, I examine the consequences of incubation temperature on incubation period, hatchling size, and sex from terrapin eggs that vary in size. I expect the warmer incubation temperatures to produce smaller hatchlings with a shorter incubation period than from cooler incubation temperatures, however the larger eggs are likely to produce larger hatchlings at all incubation temperatures. This species has temperature-dependent sex determination (TSD) so I expect male hatchlings to be produced at low temperatures, females to be produced at warm temperatures, and a population-specific sex ratio at intermediate temperatures (Ewert, 2005). Additionally, I test the following assumptions of the energetic constraint hypothesis: 1) natural nest temperatures decrease with latitude, such that eggs in southern populations are exposed to warmer incubation temperatures than eggs in northern populations and 2) eggs collected from southern populations require more yolk energy to complete development than eggs collected from northern populations.

Materials and Methods

Egg Collection and Incubation

I conducted nesting surveys during the 2004 nesting season on Grice Island, South Carolina (32°47'N, 79°56'W), Patuxent River, Maryland (38°27'N, 76°39'W), and Nokum Hill, Rhode Island (41°45N, 71°31'W). Nests that were under 12 hours of age,

determined by fresh tracks and the presence of unchalked eggs, were excavated and all eggs were collected. I froze a subset of eggs from each clutch to determine the previously reported yolk lipid components (Allman, Chapter 2). Because it was difficult to locate fresh nests in South Carolina, I captured gravid females and induced oviposition using oxytocin injections within 48 hours of capture (Ewert and Legler, 1978). All eggs were collected at the beginning of nesting season for each population (RI: June 20, MD: June 1, SC: May 20), thus a different female produced each clutch and the nests are most likely the first clutch of the season for that female.

The eggs were packed in sand and stored below 20° C for transport to Ohio University. An equal number of eggs from each clutch were randomly assigned to one of three Percivial Model 30-BLL incubators set at constant temperatures of 27° C, 28.5° C, and 30° C. The eggs were randomly placed in individual plastic compartments containing a vermiculite: water (1:2 ratio) substrate. The plastic containers were autoclaved and acid-washed to minimize any leaching of plastic material into the substrate. Additionally, the vermiculite was autoclaved to minimize bacterial growth during incubation. Water was added throughout the incubation period as needed to maintain equal substrate moisture levels at all treatments. The egg containers were rotated twice each week to minimize the potential effects of small temperature gradients within the incubator. I measured the mass and carapace length of all hatchlings within two days of emerging from the egg. An ANCOVA (SAS version 9.1.3, SAS Institute, Cary, NC) was used to determine if incubation period, hatchling mass, or hatchling-carapace length varied among treatment groups. Wet egg mass was used as the covariate with source population, incubation temperature, clutch, and sex entered as the

independent variables. All interactions were explored but to increase statistical power only the significant variables were included in the final model (Sokal and Rohlf, 1995). I used a Tukey-Kramer multiple comparison post hoc test to determine the relationship among individual groups. In all figures, I present the least square means of the dependent variable which is mathematically adjusted for egg size.

Substrate Temperatures

To determine natural incubation temperatures, five ibutton data loggers (Maxim Dallas Semiconductor, Sunnyvale, CA) were placed at nest depth on the nesting beach at each site. Ambient temperature was measured from June 1 through August 31 at 75 minute intervals. Daily mean temperatures were plotted and a repeated measures ANCOVA (SAS version 9.1.3, SAS Institute, Cary, NC) was used to test for population differences in natural incubation temperatures.

Hatchling Energetics

Upon hatching, 45 hatchlings, representing five individuals from each population and incubation temperature, were sacrificed for whole body lipid analysis. These hatchlings were dried to a constant mass at 60° C and then ground to an even particle size. The sample was transferred to a new pre-weighed Whatman cellulose thimble (33 X 80 mm) and then placed into a SoxTech HT2 1045 extraction unit. The calculated sample recoveries after transferring to the extraction thimbles range from 96% to 99%. Petroleum ether was refluxed through the samples for 90 minutes at 100° C, the samples were then rinsed for 45 minutes, and the remaining solvent was evaporated for an additional 15 minutes. The extracted lipids were dried in a drying oven at 60° C for 60

minutes before weighing to the nearest 0.0001 g. I extracted six empty extraction thimbles to serve as blank controls. The amount of lipids utilized during incubation was calculated by subtracting the hatchling lipid mass from the previously reported yolk lipid mass from these clutches (Allman, Chapter 2). I used an ANCOVA (SAS version 9.1.3, SAS Institute, Cary, NC) to determine if differences existed in hatchling lipids among the population source and incubation temperature. Lean hatchling dry mass was used as the covariate and all interaction terms were explored, but only the significant interactions were kept in the final model. A Tukey-Kramer multiple comparison post hoc test was used to determine the relationship among individual groups.

Hatchling Sex

At seven months of age, the sex of each hatchling was determined using a non-lethal laparoscope technique described by Rostal et al. (1994). After applying an antibiotic treatment, I made a stab incision into the inguinal region cranial to the hindlimb. The serosa was perforated and a 2.0 mm OD (0° angle) endoscope (#27017A, Karl Storz Endoscopy, America Inc, Culver City, California) was used to view the gonad within the body cavity. Twenty hatchlings were sacrificed to validate the technique. I was 100% accurate at determining the sex of the hatchlings using the laparoscopy procedure, and there was a 100% survival rate two months after the procedure. A few hatchlings died before determining the sex so for these individuals, sex was determined by gross analysis of the gonad. Sex ratios were determined for each population at each incubation temperature to determine if this species exhibits geographic variation in the pattern of temperature-dependent sex determination (Ewert et al. 2005). I used a

heterogeneity G-test (Sokal and Rohlf, 1995) to test if the sex ratios were homogeneous and could have come from a single population.

Results

The mean substrate temperature varied over time (RANCOVA, $F_{91, 552}$=2.5, P<0.0001) and was different among each population (RANCOVA, $F_{2, 552}$=69.3, P<0.0001) (Figure 3.1). However there was an interaction effect with population and time (RANCOVA, $F_{182, 552}$=1.29, P=0.029). The mean substrate temperature in South Carolina was 30.1° C (±0.19) whereas the mean substrate temperature in Rhode Island was 23.5° C (±0.23). The highest daily mean temperature in the Rhode Island substrate was 27.3° C, however the daily mean temperature in South Carolina reached 33.3° C. As expected, the substrate temperature exhibited a latitudinal cline such that substrate temperatures increased with decreasing latitude.

Among the collected eggs, incubation period varied with incubation temperature (ANCOVA, $F_{2, 289}$=1799.2, P<0.0001) and populations (ANCOVA, $F_{2, 289}$=487.5, P<0.0001), however there was an interaction among population and incubation temperature (ANCOVA, $F_{4, 289}$=6.32, P<0.0001). For all locations, eggs incubated at 27° C required a longer incubation period than eggs from 28.5° C or 30.0° C (Figure 3.2). Likewise, for all incubation temperatures, eggs from South Carolina required a longer incubation period than eggs from Maryland or Rhode Island. The incubation period increased from 46.0 days at 30° C to 56.4 days at 27° C for eggs collected in Rhode Island, whereas the incubation period increased from 52.2 days at 30° C to 64.9 days at 27° C for eggs collected in South Carolina. Tukey-Kramer analysis indicated that incubation period varied among each incubation temperature and population treatment

(Tukey-Kramer, all comparisons, $P<0.0001$), such that incubation period decreased with increasing temperature and latitude.

Hatchling mass decreased with increasing incubation temperature (ANCOVA, $F_{2, 289}=5.2$, $P<0.0001$) for all populations, however the source population also accounted for differences in hatchling mass (ANCOVA, $F_{2, 289}=30.5$, $P<0.0001$) (Figure 3.3). For each incubation temperature, hatchling mass increased with decreasing latitude (Tukey-Kramer, all comparisons, $P<0.0001$). Eggs from Rhode Island incubated at 27° C produced hatchlings with a mean mass of 7.4 g whereas a 30.0° C incubation temperature produced hatchlings with a mean mass of 6.9 g. Additionally, eggs from South Carolina incubated at 27° C produced hatchlings with a mean mass of 8.9 grams whereas at 30° C, hatchlings had a mean mass of 8.0g. Hatchling carapace length also varied with incubation temperature (ANCOVA, $F_{2, 289}=16.1$, $P<0.0001$) and population (ANCOVA, $F_{2, 289}=27.5$, $P<0.0001$). However, Tukey-Kramer analyses indicated no differences in carapace length among hatchlings from 27° C and 28.5° C (Tukey-Kramer, q= 2.3, $P=0.1203$). Hatchling carapace length, for all incubation temperatures, increased with decreasing latitude (Tukey-Kramer, location comparisons, $P<0.0001$). Thus, hatchling mass and carapace length decreased with increasing incubation temperature and the hatchling size at any temperature varied with population source (Figure 3.3).

After accounting for body size differences (ANCOVA covariate, $F_{2, 40}=15.4$, $P=0.0004$), the amount of lipids available to the hatchlings varied with population (ANCOVA, $F_{2, 40}=57.1$, $P<0.0001$) and incubation temperature (ANCOVA, $F_{2, 40}=5.8$, $P=0.007$) (Figure 3.4). Hatchlings from Rhode Island eggs incubated at 27° C contained 0.305 g of lipid (78% of egg lipids), whereas hatchlings from 30° C contained 0.348 g of

lipid (92% of egg lipids). Similarly, hatchlings from South Carolina eggs that were incubated at 27° C contained 0.44 g (67% of egg lipids) and eggs from 30° C contained 0.49 g (74% of egg lipids) of lipids. At each incubation temperature, the South Carolina eggs utilized a larger amount of egg yolk than other populations. The amount of lipid utilized during embryogenesis varied among populations (ANCOVA, $F_{2, 40}$=622.4, P<0.0001) and incubation temperature (ANCOVA, $F_{2, 40}$=73.0, P<0.0001). The embryos from Rhode Island utilized less lipids during development than other populations at all three incubation temperatures and lipid decreased with increasing incubation temperatures (Tukey-Kramer, all comparisons, P<0.0001) (Figure 3.4). These data indicate that more energy is required to complete embryogenesis at cooler incubation temperatures and that embryos in South Carolina eggs utilized more energy to complete development than embryos in eggs from northern populations. However, because the eggs from South Carolina contained a larger amount of yolk lipids, the hatchlings from South Carolina had a higher amount of NPL energy stores at all incubation temperatures.

Eggs from each population incubated at 27.0° C produced 100% male hatchlings, whereas all hatchlings from eggs incubated at 30° C were 100% female (Table 3.1). The 28.5° C incubation temperature produced mixed sex ratios that varied among populations (Heterogeneity G-test, χ^2=19.8, P<0.0001); suggesting the pivotal temperature is shifted toward a lower temperature in the RI population.

Discussion

The aim of this study was to develop a new understanding of intraspecific egg size variation exhibited in oviparous ectotherms. I examined the consequences of incubation temperature on incubation period, hatchling size, and sex from terrapin eggs

collected from three populations. I expected the warmer incubation temperatures to produce smaller hatchlings with a shorter incubation period than cooler incubation temperatures. Also, the larger eggs were expected to produce larger hatchlings at all incubation temperatures. Additionally, I tested the following assumptions of the energetic constraint hypothesis: 1) natural nest temperatures decrease with latitude, such that eggs in southern populations are exposed to warmer incubation temperatures than eggs in northern populations and 2) eggs collected from southern populations use more yolk energy to complete development than eggs collected from northern populations. To meet these assumptions, I expected the nest-site substrate temperatures to increase with decreasing latitude and that eggs from southern populations would require more energy to complete development than eggs from northern populations.

Variation in egg size is common in wide-ranging oviparous ectotherms (Sinervo, 1990; Iverson et al; 1993). This variation results from a plastic response of a single genotype (phenotypic plasticity) or through local adaptation to specific sets of environmental conditions (Stearns, 1989; Reznick, 1996). In either case, the mechanistic link between the environment and the expressed phenotype is essential to understanding the diversification of life histories (Ricklefs and Wikelski, 2002).

The evolution of offspring number and size is mediated by balancing the advantage of fecundity and offspring survival, under the assumption that offspring fitness increases with size. Therefore, variation in maternal investment not only affects the offspring size, but will also influence offspring traits such as metabolism, performance, and growth. By experimentally manipulating egg size, Sinervo (1990) demonstrated that variation in *Sceloporus occidentalis* offspring fitness traits resulted from variation in

maternal investment to each offspring. Although there is a growing body of literature suggesting larger offspring are more fit (Ferguson and Fox, 1984; Janzen et al. 2000), small offspring do not necessarily have reduced fitness in all environments (Sinervo, 1990).

In *Malaclemys terrapin*, females in southern populations deposit larger eggs that contain a higher proportion of energy stores than females in northern populations that deposit smaller eggs (Allman, Chapter 2). The nest-site substrate temperatures indicate eggs deposited in southern climates would likely have higher incubation temperatures than eggs in northern populations. At each incubation temperature, and after correcting for variation in egg size, eggs collected from South Carolina required a longer incubation period and produced larger hatchlings than eggs collected from Maryland or Rhode Island. Additionally, the incubation temperature influenced the amount of energy utilized during embryogenesis and the amount of energy stores remaining after hatching. If the latter reserve provides a fitness advantage to the offspring, then it is likely that the energetic demand of the hatchling may explain latitudinal variation in egg size as predicted from the energetic constraint hypothesis. For example, in southern climates, females that build large eggs may be favored due to the increased energy required to complete development in this population.

Hatchling energy stores are not the only influence incubation temperature has on hatchling phenotypes. The thermal properties during development also influence hatchling parameters such as incubation period, size, performance, metabolic rate, and even heart rate (Burger, 1990; Allsteadt and Lang, 1995; Shine and Harlow, 1996; Birchard, 2000). These parameters can affect hatchling survivorship, therefore

influencing the evolutionary fitness of offspring and parent. Optimal egg size theory predicts that females maximize maternal fitness and offspring fitness by producing an egg size appropriate for the local environmental conditions (Smith and Fretwell, 1974; Brockelman, 1975). Therefore, it follows that egg size variation is expected among populations exposed to different climate conditions, and that the appropriate amount of resources are allocated to eggs exposed to the local thermal properties.

A considerable number of theoretical explanations have been extended to explain latitude variation in offspring size (reviewed in Iverson et al. 1993), however very little empirical support has been established for any given hypothesis. Each of these hypotheses attempts to link selection on offspring size with hatchling fitness measures such as growth or survivorship. For example, Moll (1979) suggested the variation in egg size might result as a demographic tradeoff in which females in northern populations produce more small eggs to compensate for increased hatchling mortality. This argument assumes hatchlings in northern populations have lower survivorship and that increasing offspring size would not increase survivorship. Although these assumptions are very difficult to test, and there has been no comparison of hatchling growth or survivorship for nests of different sizes across latitudes, recent work has indicated larger offspring have increased survivorship (Ferguson et al. 1982; Janzen et al. 2000; Räsänen et al. 2005; but see Congdon et al. 1999). Therefore, if larger hatchlings have higher survivorship in northern populations then selection is expected to increase egg size instead of clutch size (Ricklefs, 1980; Iverson et al. 1993).

Alternatively, the incubation environment may act as a selection agent for variation in egg size. The temperature, water potential, oxygen levels, and soil

characteristics of the nesting substrate can effect embryonic development, hatchling phenotypes (Packard, 1999) and hatchling fitness (Janzen et al. 2000). Differences in rainfall among populations may alter the amount of water available during embryonic development, and may therefore influence egg size so that eggs laid in dryer conditions have more water to prevent desiccation. This hypothesis did not explain egg component variation among populations of snapping turtles (Finkler et al. 2004) and the proportion of water content does not vary with egg size among populations of *Malaclemys terrapins* (Allman, Chapter 2).

A similar hypothesis is that incubation or post-hatching temperature may influence egg size and explain egg size variation among populations. Variation in egg components among populations of snapping turtles was recently explained through the effects that environmental temperatures have on neonatal energy consumption (Finkler et al., 2004). If the incubation temperature or post-hatching temperature increases the energetic demand to complete embryogenesis or for neonate metabolic costs, then eggs that contain a larger amount of energy stores may produce hatchlings that exhibit increased fitness traits. The hatchlings from South Carolina utilized a larger proportion of the available energy during embryogenesis, however due to the larger maternal investment provided to these eggs, the neonate had a larger amount of energy stores available at hatching. The recorded sand temperatures indicated incubation temperatures are higher for eggs in southern populations. These higher incubation temperatures result in smaller hatchlings that utilize less energy to complete development. This suggests that the effect incubation temperature has on embryonic energy utilization does not explain the variation in egg size and energy stores exhibited *Malaclemys terrapin*. However, the

patterns of energy utilization and nest temperatures meet two of the assumptions implied in the energetic constraint hypothesis.

Populations of the diamondback terrapin exhibit variation in life history traits (Seigal, 1980; Roosenburg, 1994) that commonly follow a latitudinal cline associated with environmental temperatures. For example, females in southern populations begin nesting earlier in the year than females in northern populations, however females from the populations in RI, MD, and SC nests two or three times within a single season (Goodwin, 1994; Roosenburg, 1994; Owens, pers com). Hatchling terrapins most likely do not eat in the wild until the following spring after emergence (Brennessel, 2006). Hatchlings in SC hatch and emerge earlier than individuals in northern populations that will often overwinter inside the nest. Therefore, hatchlings in southern populations are active for a longer period of time before feeding and may require a larger amount of energy reserves to survive through the winter into the spring. It follows that maternal investment to individual offspring may influence offspring fitness through the energetic requirement of hatchlings during the neonate period of a negative energy budget. However, to explicitly test the energetic constraint hypothesis, offspring metabolism, growth, and survivorship need to be described from different populations in a common-garden style experiment.

The low incubation temperature produced 100% male hatchlings from all populations and high incubation temperatures produced 100% female hatchlings from all populations. Interestingly, the percentage of male hatchlings produced at the intermediate incubation temperature was higher from South Carolina eggs than Rhode Island eggs. These data suggest population-specific pivotal temperatures that decrease

with increasing latitude. In wide ranging species with temperature-dependent sex determination, the pivotal temperatures or maternal nest site selection is expected to vary according to local climate (Bulmer and Bull, 1982; Bull, 1983; Rhen and Lang, 1998; Morjan, 2003; Ewert et al. 2005). Variation in pivotal temperatures has been described for several species including *Graptemys pseudogeographica*, *Chrysemys picta*, and *Chelydra serpentina* (Bull et al. 1982, Ewert et al. 1994). However, the variation in pivotal temperature is not large enough to compensate for the differences in natural incubation conditions among populations. Therefore, it is likely that maternal nest site selection for warmer or cooler microclimate conditions may be important in maintaining a balanced sex-ratio. Roosenburg (1996) described the maternal effect of nest site choice on offspring sex ratio in the diamondback terrapin.

Although temperature is the primary determinant of hatchling sex in TSD species, the quantity of yolk steroid hormones has been correlated with variation in sex ratios within a nesting season (Bowden et al. 2000). In a population of *Chrysemys picta*, the hatchling sex ratio shifted from a male to a female biased sex ratio as yolk estradial levels increased (Bowden et al. 2000). If maternally derived estrogen levels influence sex determination, then perhaps the increased proportion of females from South Carolina eggs can be explained by the presence of more estradial in these larger eggs. Preliminary studies have indicated there is no difference in estradial levels among diamondback terrapin eggs collected from Rhode Island, Maryland, and South Carolina (Allman et al. 2005), however this needs further investigation with a larger sample size.

In summary, I have demonstrated population specific variation in hatchling phenotypes at different incubation temperatures in the diamondback terrapin. Optimal

egg size theory predicts that balancing selection among fecundity and offspring size will simultaneously maximize maternal and offspring fitness within a given environment (Smith and Fretwell, 1974). In wide-ranging species that are exposed to different environmental conditions, the optimal egg size is expected to vary if environmental conditions influence the relationship between offspring size and fitness. This paper demonstrates the incubation environment influences hatchling phenotypes in a population-specific manner. Furthermore, since hatchlings in southern populations are exposed to higher incubation temperatures and utilize more energy to complete development, I have confirmed the two assumptions underlying the energetic constraint hypothesis. However, this hypothesis also assumes that hatchlings with more energy stores will have a growth or survival advantage over hatchlings with less energy stores in southern climates. Additionally, the fitness advantage of large offspring is expected to be reduced in northern climates such that maternal fitness is maximized through fecundity instead of offspring fitness. These additional assumptions need to be addressed in the diamondback terrapin and other wide-ranging oviparous ectotherms.

Chapter 4: Experimental analysis of hatchling energetics and fitness traits among three populations of the diamondback terrapin, *Malaclemys terrapin*

Phil Allman and Willem Roosenburg

Introduction

Understanding the relative influence of genetic and environmental effects on intraspecific phenotypic variation remains at the core of life history research. Phenotypic variation in life history traits such as fecundity, offspring size, growth and survivorship have been the target of empirical and theoretical studies attempting to understand the coevolution of these traits (Roff, 1992; Stearns, 1992). Species with a wide geographic range have long been at the core of this research because adaptation to local environmental conditions is expected (Sinervo, 1993; Reznick et al. 1997; Huey et al. 2000). Despite numerous studies that provide correlative support for local adaptation, few identify the actual physiological and demographic mechanisms that underlie the traits that collectively result in the local life history phenotype. Reaching a full understanding of intraspecific variation in parental investment requires a complete examination of reproductive output and the influence of that output on offspring fitness. In this study, I test the energetic constraint hypothesis that has been proposed to explain egg size variation among populations of a single species (Allman, Chapter 2) by investigating how variation in egg size and incubation temperature influences hatchling energetics and fitness traits among different populations.

Life history theory predicts that the number and size of offspring produced should optimize maternal fitness (Lack, 1947). However, since energy available for reproduction is limited, a tradeoff between number and size of offspring occur (Smith and Fretwell, 1974; Brockelman, 1975). Therefore, an optimal egg size may exist where the female and offspring fitness are simultaneously optimized. When initial egg size determines offspring size and performance, different reproductive strategies are expected

among different environmental conditions (Smith and Fretwell, 1974; Brockelman, 1975; Hendry et al. 2001). For example, investment in a small number of large eggs is expected when larger offspring size improves future offspring fitness.

Oviparous reptiles serve as a good model system to investigate the influence of environmental heterogeneity and egg size on offspring phenotype. With the exception of the energy used for choosing a nest site, reptile eggs contain the total energy allocated by the female to each offspring. Parental investment in an egg provides for the complete development of a hatchling (PIE), and investment in care of the hatchling (PIC) (Congdon, 1989). Residual yolk and hatchling fat reserves constitute PIC that is used for post-hatching maintenance and growth (Congdon, 1998). The egg represents the majority of the female's reproductive investment and larger eggs typically represent a larger maternal investment (Ricklefs and Burger, 1977; Congdon et al. 1983a; Sinervo, 1993; Roosenburg and Dennis, 2005). Most reptiles, particularly turtles, have large among-clutch variation in egg size and composition; however eggs within a clutch vary little in size and composition (Finkler et al. 1997; Roosenburg and Dunham, 1997; Roosenburg and Dennis, 2005).

In addition to the influence maternal investment has on offspring phenotype, the embryonic environment can influence offspring phenotypes such as size, sex, and energy stores (Yntema, 1978; Packard et al. 1983; Gutzke and Packard, 1987; Congdon and Gibbons, 1989; Deeming and Furguson, 1991; Van Dame et al. 1992). For example, low incubation temperatures reduce metabolic rate, increase incubation time, and produce larger hatchlings (Christian et al. 1986; Aulie et al. 1989; Deeming and Ferguson, 1989; Leshem et al. 1991; Andrews et al. 1997; Booth, 1998; Booth et al. 2000; Booth and

Astill, 2001). The total energetic cost of development usually increases in such situations and results in slower growth rates (Allsteadt and Lang, 1995; Rhen and Lang, 1999). In wide ranging species, the incubation temperature and post-hatching environment can vary greatly and thus result in different patterns of maternal lipid allocation to eggs, embryo lipid utilization during development, and subsequently hatchling size (Allman, Chapter 3). Because egg size is influenced by maternal investment, when egg size affects hatchling fitness, among-population variation in maternal investment may reflect adaptive responses to local environmental conditions.

The diamondback terrapin is a wide-ranging emydid turtle that has a semi-continuous range from the northeast United States to the Texas coast. This species exhibits geographic variation in egg size (Seigel, 1984; Allman et al., 2003) and composition (Allman, Chapter 2). Although adult females are larger in Northern populations (Montevecchi and Burger, 1975), they deposit smaller eggs that contain less energy for the developing embryo (Allman, Chapter 2). In contrast, female terrapins in southern populations deposit larger eggs that contain a higher proportion of energy. Additionally, hatchlings from southern populations have higher energy stores after hatching than hatchlings from northern populations (Allman, Chapter 3), however it is unknown if this variation results in fitness differences among offspring of different populations. The energetic constraint hypothesis assumes that hatchlings in southern environments are exposed to conditions that increase their metabolic energy demand, thus requiring a larger amount of energy stores to grow and survive in those conditions. Furthermore, hatchlings in southern populations emerge earlier and have an extended period of activity compared to hatchlings in northern populations. In the present study, I

test two assumptions of the "energetic constraint" hypothesis by investigating 1) if the incubation conditions eggs in southern populations are exposed to result in hatchlings with a higher energetic demand, and 2) if hatchlings from a southern population have higher growth and survivorship than hatchlings from northern populations raised in a common environment.

Materials and Methods

Egg Collection and Incubation

Freshly deposited diamondback terrapin, *Malaclemys terrapin*, eggs were collected on Grice Island, South Carolina (32°47'N, 79°56'W), Patuxent River, Maryland (38°27'N, 76°39'W), and Nokum Hill, Rhode Island (41°74N, 71°31'W). Eggs deposited within a 12 hour period, determined by amount of chalking, were excavated and stored at 18° C for transporting to Ohio University. Because it was difficult to locate fresh nests in South Carolina, I captured gravid females and induced oviposition using oxytocin injections within 48 hours of capture (Ewert and Legler, 1978). All eggs were collected at the beginning of nesting season for each population, thus a different female produced each clutch and the nests most likely represent the first clutch of the season for that female.

An equal number of eggs from each clutch were randomly assigned to one of three Percivial Model 30-BLL incubators set at constant temperatures of 27° C (RI: n=45, MD: n=34, SC: n=22), 28.5° C (RI: n=45, MD: n=34, SC: n=22), and 30° C (RI: n=45, MD: n=34, SC: n=22). The eggs were incubated in a vermiculite: water (1:2 ratio) substrate. Water was added throughout the incubation period as needed to maintain equal substrate moisture levels at all treatments. I rotated the egg containers each week to

minimize the effects of any temperature gradient within the incubator. Upon hatching, mass (to the nearest 0.1 g) was recorded and a unique set of marginal scutes were notched to identify individuals.

Hatchling Oxygen Consumption

The oxygen consumption of hatchlings from each population and incubation temperature [27° C: (RI: n=38, MD: n=26, SC: n=16); 28.5° C: (RI: n=28, MD: n=15, SC: n=13); 30° C: (RI: n=36, MD: n=21, SC: n=11) was measured using a Sable System closed system respirometry system. Six individuals were measured simultaneously with a baseline chamber in a Percivial Model 30-BLL incubation chamber set at a constant temperature of 28.5° C. Following the absorption of the yolk plug into the shell (3-6 days after pipping), the hatchlings were placed in individual 100 ml glass metabolic chambers four hours prior to data collection. This time allowed the hatchlings to acclimate to the temperature and recover from any handling stress caused by placing them in the chambers. The amount of oxygen consumed during a one-hour period was measured during the quiescent period of scotophase.

In succession, each metabolic chamber received dry, carbon dioxide-free ambient air at a rate of 100 ml/minute for seven minutes. Datacan (Sable Systems, Inc.) was used to ensure each chamber was thoroughly flushed during the seven-minute period. Each chamber was then closed for 60 minutes before flushing again for seven minutes. I determined the total amount of oxygen consumed during the 60 minute period by integrating total oxygen levels and adjusting for flow rate using Datacan. The procedure was repeated six times for each hatchling to calculate mean O_2 consumption. An ANCOVA with mean O_2 consumption as the dependent variable; population source,

incubation temperature, and sex as independent variables; and body mass as the covariate was used to determine differences among the treatment groups. A Tukey-Kramer multiple comparison post hoc test was used to determine the relationship among individual groups.

Hatchling Growth and Survivorship

After measuring O_2 consumption, I randomly assigned hatchlings from different populations (RI: n=104, MD: n=75, SC: n=53) and incubation temperatures (27° C: n= 91, 28.5° C: n=68, 30° C: n=83) to one of three growth treatment rooms. These rooms mimicked the water temperature and light cycle for the three source populations in RI (cold room), MD (warm room), and SC (hot room) (Figure 4.1). The water temperature and light cycle was adjusted every 15 days to mimic the light cycle and the ten-year mean water temperature for that location at that time period (NODC/NOAA, 2004). Each turtle was raised in an acid-washed plastic housing unit (35 cm L X 19 cm W X 11 cm D) containing water at 10 ppt salinity. Hatchlings were fed a commercial turtle chow (Nasco Biologicals, Fort Atkinson, Wisconsin) *ab libitum* twice per week and water changes were conducted 24 hours after each feeding. The containers were rotated twice per week to account for small variations in temperature within the growth rooms. Every 30 days, I measured hatchling mass to the nearest 0.1 g. I used two repeated measures ANOVAs with hatchling mass and change in mass as the dependent variables to determine differences among population, growth treatment, sex, and incubation temperature. I developed a full model exploring all interaction affects, but only kept the significant variables in the reduced model.

Results

Incubation temperature increased oxygen consumption in hatchlings from all populations (ANCOVA, $F_{2, 203}$=187.7, P<0.0001) (Table 4.1). However an interaction effect among incubation temperature and population (ANCOVA, $F_{4, 203}$=182.4, P<0.0001) indicates that the incubation temperature effect on hatchling metabolism differed among the three populations (Figure 4.2). For all incubation temperatures, hatchlings from South Carolina eggs had a higher mass-specific metabolic rate than hatchlings from other populations (ANCOVA, $F_{2, 203}$=179.8, P<0.0001). Tukey-Kramer analysis indicated that oxygen consumption varied among each incubation temperature and population treatment (Tukey-Kramer, all comparisons, P<0.0001), such that oxygen consumption increased with incubation temperature and decreased with increasing latitude.

Hatchling Growth and Survivorship

At the beginning of the growth experiment, hatchlings from the same population assigned to different growth treatments did not differ in mass (ANCOVA, $F_{4, 230}$=0.84, P=0.49) and carapace length (ANCOVA, $F_{4,230}$=1.1, P=0.35). For all growth treatments, the reduction in temperature in the first three months caused the hatchlings to stop eating. Hatchlings in the cold and warm growth room were inactive by month four although hatchlings in the hot growth room stayed active for the entire 6-month period. The incubation temperature did not influence changes in hatchling mass (RANCOVA, $F_{2, 215}$=0.25, P=0.78) over the six-month period so hatchlings from different incubation temperatures were grouped together for analyses.

Source population (RANCOVA, $F_{2, 215}$=31.9, P<0.001) and the growth environment (RANCOVA, $F_{2, 215}$=139, P<0.001) influenced the pattern of hatchling mass change over the six month growth period (Table 4.2). However an interaction among population source and growth environment (RANCOVA, $F_{4, 215}$=13.1, P<0.001), indicated the environmental influence (growth environment) on growth depended on the source population. Hatchlings from SC eggs in the hot growth room were the largest at the end of the 6-month period, followed by hatchlings from MD eggs in the hot growth room. These two groups were the only two that had a net gain in hatchling mass after the six-month period (Figure 4.3). For all populations, hatchlings grown in the hot growth room were larger than hatchlings from the same population grown in the other growth rooms. The rate of growth increased with increasing growth room temperature (RANCOVA, $F_{2, 215}$=167, P<0.001), and varied among populations (RANCOVA, $F_{4, 215}$=46.6, P<0.001) (Figure 4.4). After the fourth month of growth, RI and MD hatchlings in the hot growth room had the highest rate of mass loss (RANCOVA, $F_{4, 84}$=10.4, P<0.001), while at the same time, the SC hatchlings in the hot growth room had the smallest rate of mass decrease among all growth rooms. These data indicate that hatchlings raised in warmer temperatures grew to a larger size than hatchlings in cooler environments; however the effect of environmental temperature was dependent on the population source of the eggs. Additionally, when the environmental temperatures decreased in the fall, hatchlings stopped eating and began losing weight at a rate that was dependent on the population source and environmental temperature.

Survival probability curves indicate differences in survival among treatment groups during the 6-month growth study (Logrank χ^2=18.6, d.f.=4,P=0.017) with very

little mortality before four months (Figure 4.5). After four months, 17% (n=5) hatchlings from Rhode Island in the hot treatment room died along with 14% (n=4) in the warm treatment room. In the cold growth room, survivorship was high with only a single individual from Rhode Island and South Carolina dying during the six month of the study. These data indicate that hatchlings from Rhode Island have a lower survivorship when exposed to warmer environmental conditions than hatchings from the other populations.

Discussion

In this study, I examined two assumptions underlying the energetic constraint hypothesis as an explanation of intraspecific variation in egg size exhibited in many oviparous reptiles. First, I demonstrated the assumption that warmer incubation temperatures, as found in southern populations, produce hatchlings that have a higher mass-specific resting metabolic rate, thus a higher energetic demand for maintenance functions immediately after hatching. Hatchlings from each population had a higher rate of O_2 consumption when eggs were incubated at warmer temperatures. At all incubation temperatures, hatchlings from South Carolina eggs had a higher mass-specific metabolic rate than hatchlings from Maryland or Rhode Island. Additionally, hatchlings from the lowest incubation temperature had the lowest mass-specific metabolic rate. I also tested the assumption that hatchlings have the highest growth and survivorship in their native environmental conditions for six months after hatching. Hatchling growth varied among population source and growth treatment, but did not vary with sex or incubation temperature. Hatchlings in the hot growth room were active throughout the experiment; however, hatchlings from RI and MD lost the most mass during this time. The rate of

mass change indicated a growth advantage in the hot growth room for hatchlings with more energy stores. In contrast, the rate of growth did not vary among population source in the cold room, suggesting larger offspring are not afforded a growth advantage in the cold environment. Survivorship analysis revealed high survivorship for all hatchlings in their native growth treatment, however survivorship of RI hatchlings began declining after four months in the hot growth room. The growth and survivorship data indicate hatchlings from northern populations would exhibit higher mortality rates in southern environments.

Metabolic Rate

Maintenance metabolism constitutes a large portion of an animal's energy budget, and in nature can make up the majority of an organism's annual energy expenditure (Congdon et al. 1982; Beaupre, 1996). The amount of available energy to fuel such processes as maintenance, growth, reproduction, and digestion may influence allocation decisions made toward each component (Dunham et al. 1989; Niewiarowski, 2001). Additionally, because some components such as maintenance metabolism are affected by temperature, abiotic environmental conditions can be a major determinant of the quantity of energy available for growth and reproduction (Dunham et al. 1989). Standard metabolic rate (SMR) is commonly used as a measure of maintenance metabolism (Bennett and Dawson, 1976), however the minimal rate of metabolism in a post-absorptive, resting animal is unlikely to be a realistic estimate of the maintenance costs imposed on free-ranging ectotherms (Waldschmidt et al. 1987; Niewiarowski and Waldschmidt, 1992). In this study, I present the resting metabolic rate (RMR) (Congdon et al. 1982) of neonate hatchlings that are not feeding, but may be assimilating residual

yolk. Because hatchling turtles are unlikely to begin feeding immediately after hatching, and will utilize energy stores as needed for maintenance, I feel this is a more ecologically relevant estimate of maintenance metabolism (Anderson and Karasov, 1981). Because the hatchlings cannot be considered fasting until the residual yolk is completely utilized, I can not directly compare these results with published SMR measurements using fasting organisms (Niewiarowski and Waldschmidt, 1992; Litgus and Hopkins, 2003).

I have demonstrated that hatchling oxygen consumption increases with incubation temperature, however hatchling mass and population source also contributed to the variation in resting metabolic rates. The interaction among population source and incubation temperature indicates oxygen consumption is a complex function that cannot be predicted without considering the population source. Clutch source was not a significant effect in explaining the variation in the RMR of the hatchlings in this study. Many authors have reported significant clutch effects on metabolic rates (Garland and Bennett, 1990; Steyermark and Spotila, 2000), however the experimental design used in this study minimized clutch effects by distributing eggs across different treatment groups. Therefore, the lack of a clutch effect is most likely reflects a lack of statistical power due to the experimental design. The small clutch sizes in *Malaclemys terrapin* (Allman, Chapter 2) makes it difficult to identify clutch effects. I also did not find a difference among the sexes in metabolic rate. The diamondback terrapin possesses temperature-dependent sex determination where male hatchlings are produced at cool temperatures and female hatchlings are produced at warmer temperatures (Jeyasuria et al. 1994). Therefore, it is difficult to separate effects of sex from that of temperature. However, eggs were incubated at three temperatures, with a middle temperature that produced a

mixed sex ratio (Allman, Chapter 3). This design allows me to separate the effects of incubation temperature from hatchling sex. Some ectotherms show sex specific metabolic rates (Ryan and Hopkins, 2000), however other species do not (Beaupre et al. 1993; Zaidan, 2003).

Intraspecific variation in metabolic rates has been demonstrated for most vertebrate ectotherms (reviewed in Garland and Adolph, 1991). For the tropical skink *Mabuya striata*, high-elevation individuals can acclimate their RMR to temperature, however low-elevation individuals cannot (Patterson, 1984). Differences in climate conditions among populations explained seasonal and annual variation in maintenance metabolism of mottled rock rattlesnakes, *Crotalus lepidus* (Beaupre, 1996). Individuals at lower latitudes or altitudes usually experience warmer temperatures throughout the year (Beaupre, 1995), therefore this variation explains, at least in part, the geographic variation in life history traits exhibited in this species. Likewise, *Malaclemys terrapin* exhibits geographic variation in life history traits. Female terrapins in southern populations produce eggs that are larger and contain a higher portion of energetic lipids (Allman, Chapter 2), which results in hatchlings with more lipid stores (Allman, Chapter 3). These larger eggs are deposited in substrates that maintain a higher temperature than substrates from northern populations (Allman, Chapter 3). Therefore, it is likely that hatchlings produced from the warmer nests in southern populations have a higher RMR, thus a higher energetic demand for maintenance metabolism immediately after hatching.

The pattern of RMR demonstrated in this study supports the assumption of the energetic constraint hypothesis that hatchlings produced from eggs in southern climates have a higher mass-specific metabolic rate than hatchlings produced from cooler

climates. However, since egg size increases with decreasing latitudes, it is difficult to separate the confounding effects of population source and egg size on the metabolic rates. I used ANCOVA to account for variation in egg size, but it would be valuable to manipulate egg size to experimentally separate the variation associated with egg size from the variation that represents a population effect (Sinervo, 1990). Additionally, because adult females were not placed in a common-garden experiment, it is difficult to say if egg size variation and resulting hatchling energetics is a consequence of phenotypic plasticity or if the variation represents an evolutionary response to different climatic conditions. Egg size is a heritable trait in several species of birds (Noordwijk et al. 1980; Moss and Watson, 1982), however the heritability of egg size in reptiles has not been thoroughly explored.

Hatchling Growth and Survivorship

Biological fitness is usually defined as the relative success of an organism to contribute its genes to the next generation and is often explained in terms of lifetime reproductive success. Unfortunately, fitness is difficult to measure and one often relies on measuring traits that are assumed to influence an organism's ability to reach maturity and contribute to the gene pool (Kozlowski, 1996; Fairbairn and Reeve, 2001). Measurements of individual quality, such as size, growth, and survivorship, can be used to infer fitness (Reznick and Travis, 2001).

In the current study, I used a common-environment experimental design to measure growth and survivorship of terrapin hatchlings produced from eggs collected at three populations and incubated at different temperatures. Unlike most studies, my growth environments mimicked the local conditions of the three source populations from

late summer to mid-winter. The light cycle and water temperature were adjusted so hatchlings would experience a similar abiotic environment as the natural environment provides in these locations.

Hatchling growth patterns varied among populations and growth environments, however an interaction effect indicates that the growth environment influenced hatchling growth differently, depending on the source population of the hatchlings. The egg incubation temperature did not affect growth pattern or growth rate over the six month period. Steyermark and Spotila (2001) reported a strong clutch effect for growth rates in the snapping turtle, *Chelydra serpentina*. Clutch identity did not influence growth in the current study, however a split-clutch experimental design was used to minimize clutch effects. Futhermore, the clutch size of *Malaclemys terrapin* is considerably smaller than in *Chelydra serpintina* making it more difficult to identify clutch effects in this species. The incubation temperature did not influence growth although the influence of embryonic temperature on growth rate has been reported in the terrapin from a three year growth study (Roosenburg and Kelley, 1996) and in other ectotherms (Bobyn and Brooks, 1994; Rhen and Lang, 1995). Steyermark and Spotila (2001) also reported no influence of incubation temperature and suggested the inconsistency may be due to differences in experimental design.

Hatchlings from SC and MD in the hot growth room were the only treatments that had net gain in mass at the termination of the experiment. Interestingly, the RI hatchlings in the hot growth room lost the most mass during the study and lost more mass during the final three months than any other treatment group. Additionally, the SC hatchlings in the hot room lost the least amount of mass during the final three months of the study.

For all growth rooms, turtles stopped eating the offered food when the ambient temperature fell below approximately 20° C and this occurred after only one month in the cold growth room. By the third month, all hatchlings stopped eating and were presumably meeting metabolic demands through stored energy obtained from previous feeding events and residual energy stores from the egg yolk. In the cold and warm growth rooms, hatchlings were inactive during the final three months and this resulted in only a small amount of mass loss during this time. However, in the hot growth room that mimicked the water temperature in SC, hatchlings remained active and this resulted in a higher rate of mass loss for RI and MD hatchlings than in the other growth rooms. This, combined with the survivorship data, suggests that the hatchlings from RI and MD did not have adequate energy stores to survive the extended period of activity. On the other hand, hatchlings from SC lost the least mass of all groups including those hatchlings in the cold environment that had not fed for a longer time period and that were inactive. This pattern may be explained by the SC hatchlings utilizing energetic stores remaining from the egg yolk to meet the energetic demand of the environment. Therefore, the larger size of SC hatchlings at the end of the growth study may result from the benefits provided to them through the presence of additional storage lipids.

The rate of mass loss in the cold and warm rooms were similar for all populations indicating that larger hatchling size in northern climates would not provide individuals with a growth advantage or even a size advantage after the first winter. At the end of the growth study, hatchlings from all populations in the cold and warm treatment room were of similar size. From an evolutionary standpoint, females that build large eggs in northern climates do not provide offspring with a growth advantage over hatchlings from

smaller eggs. However, in southern climates, females that build large eggs provide the offspring with a growth advantage possibly through the allocation of more energy stores that fuel the metabolic demands of hatchlings in the warmer climate. This variation in maternal investment is predicted in the energetic constraint hypothesis, demonstrating that optimal egg size varies with environment and may not necessarily select for larger offspring.

Recent work has demonstrated that maternal investment in eggs can have long-lasting consequences on offspring fitness (Einum and Fleming, 2000; McIntyre and Gooding, 2000) that can affect population dynamics over many generations (Benton et al. 2005). Maternal investment can influence growth rate, which may determine age and size at maturity, which ultimately will influence the egg size produced by that individual. Additionally, there may indirect effects such as the influence offspring size has on the individual's ability to compete for resources (Benton et al. 2005). The competitive environment can influence growth rate, size at maturity, and may ultimately affect the allocation decisions associated made by the adult (Dunham et al. 1989). In long-lived oviparous ectotherms, the production of larger offspring is thought to improve offspring survivorship due to a decreased time spent in the juvenile size class and through a competitive advantage over smaller individuals (Stearns, 1992; Janzen et al, 2000). However this 'bigger is better' hypothesis isn't supported in many systems (Fox, 1978; Fergusson and Fox, 1984; Congdon et al. 1999).

The survivorship curve indicates the potential influence environmental conditions have on hatchling fitness. For hatchlings from all populations, survivorship was 100% throughout the experiment in the warm growth room. After month four, survivorship

decreased for hatchlings from RI in the hot growth room. The survivorship for this group continued to decrease for the remainder of the experiment. Likewise, MD hatchlings followed a similar pattern in the hot growth room. These patterns indicate that hatchlings from northern populations would have reduced survivorship if placed in climate conditions similar to that of South Carolina. The source of the mortality is unknown, however it is most likely associated with size and may have resulted from the lack of resources to meet metabolic demands. Other sources of mortality may have caused the observed survivorship patterns, nonetheless the correlated patterns of growth and survivorship with the metabolic rates indicate that hatchling fitness is most likely influenced through a complex interaction among environmental, genetic, and maternal effects.

Other Considerations:

Increasing interests around global climate change has been directed toward understanding the potential impacts on physiological processes (Kareiva et al. 1993) as well as impacts to native habitat (Root et al. 2003; Huntley et al. 2004) and the possibility of extinction (Thomas et al. 2004). Already, evidence suggests that warming temperatures have resulted in changes in growing seasons (Menzel and Fabian, 1999), species ranges (Parmesan et al. 1999), and even patterns of seasonal breeding (Beebee, 1995). Warming climates may also affect an organism's energy budget and result in changes to the life history of the organism.

The current study provides insight into the mechanism by which environmental conditions can influence life history traits among populations of a single species. I have shown that environmental temperatures influence hatchling metabolic rate, growth and

survivorship. Therefore, global climate change may have direct adverse effects on the growth and survivorship of hatchling turtles in their native environments.

In *Malaclemys terrapin*, it is likely that an increase in environmental temperature will have two interrelated consequences to hatchling fitness traits: 1) substrate temperatures are likely to rise which will increase the ambient temperature embryos develop in, and 2) the water temperature is likely to rise which will increase the environmental temperature hatchlings are exposed to. The first consequence will result in the production of smaller hatchlings that have a higher maintenance metabolism thus requiring a larger amount of energy stores to fuel the increased costs. The second consequence will likely cause a higher energetic demand that will require the organism to allocate more energy to maintenance at the expense of storage or growth. Winter temperatures are expected to increase disproportionately to summer temperatures (IPCC, 2001), and are predicted to have negative impacts on other ectotherms such as red-eared sliders, *Trachemys scripta* (Willette, et al. 2005). The influence of global climate change on physiological processes that affect life history traits may be difficult to demonstrate, however these influences may have dramatic impacts for population stability.

Conclusions:

In this paper, I have demonstrated that the incubation temperature influences hatchling oxygen consumption through a population-specific interaction that results in higher metabolic rates in warmer incubation temperatures. Additionally, at all incubation temperatures, hatchling from the southern population had a higher mass-specific metabolic rate and demonstrated a reduction in metabolic rate with increasing latitude among the three populations. Hatchlings in the hot room, from all populations, were

larger at the end of the six-month study than hatchlings in the warm or cold treatment rooms. The pattern of growth demonstrates that larger hatchlings are provided a growth advantage in hot climate conditions, but this advantage is lost in cold climates. Additionally, survivorship was highest for all treatment groups in their native environmental conditions. For example, hatchlings from Rhode Island eggs had the lowest survivorship in the hot environment, whereas it was highest in the cold environment. The purpose of this study was to test the energetic constraint hypothesis by analyzing hatchling fitness traits in a common-garden experimental design. These data indicate that females in southern populations that deposit large eggs may be selected through the growth and survival advantage afforded to larger offspring, whereas females in northern populations that deposit smaller eggs may be selected through increased fecundity at no cost for offspring survivorship or growth.

Chapter 5: How quickly do turtle hatchlings burn fat? An analysis of posthatching yolk energy utilization in fasted diamondback terrapin, *Malaclemys terrapin*, raised in different environments.

Phil Allman and Willem Roosenburg

Introduction

Optimal egg size theory attempts to explain the relationship of maternal investment in egg size and number. In situations where resources are limited, a tradeoff exists between the size and number of eggs (Smith and Fretwell, 1974; Brockelman, 1975; Parker and Begon, 1986), such that an increase in egg size results in the production of fewer offspring. Thus, maternal fitness is maximized when investment to offspring simultaneously optimizes egg size and number (Smith and Fretwell, 1974).

Recently, I used the energetic constraint hypothesis to explain the geographic variation in egg size found in an oviparous reptile (Allman, Chapter 4). This hypothesis asserts that females in southern populations deposit larger eggs than females in northern populations because hatchlings in southern climates have a higher energetic demand and require larger energy stores to survive through the period of a negative energy budget into the following spring. Larger hatchlings with higher energy stores received a growth and survivorship advantage in warm conditions (Allman, Chapter 4). The reduced survivorship among the smaller hatchlings may have resulted from the lack of sufficient energy to fuel the individual's metabolic demand in the warmer conditions. However, the energy stores were not measured and there is currently no information on the rate of energy utilization in neonate turtles raised in different environments. The energetic constraint hypothesis assumes that hatchlings in warmer climates utilize residual energy reserves at a higher rate than in cooler climates. To test this assumption, I measured the rate of lipid utilization in hatchling turtles raised at temperatures mimicking a hot and a cold climate regime.

The energetic material provided to eggs by the female can be viewed as investment for embryogenesis (PIE) and extended parental care (PIC) (Congdon, 1989). Residual yolk and hatchling fat reserves constitute PIC, and are used for dispersal from the nest site and post-hatching maintenance (Kramer and Bennett, 1981; Congdon and Gibbons, 1990; Nagle et al. 1998). The amount of energy reserves available to the hatchling is determined by the influence of the incubation environment on energy utilization during embryogenesis (Congdon and Gibbons, 1990; Nagle et al. 1998). The amount of energy available when the hatchling leaves the egg typically exceeds 50% of the original energy in the egg (Congdon et al. 1983a; Wilhoft, 1986; Gutzke et al. 1987; Nagle et al. 1998; Thompson et al. 1999; Allman, Chapter 3), suggesting investment in PIC represents a substantial portion of the maternal investment to individual offspring.

In some situations, hatchlings that contain more energy reserves may have a fitness advantage compared to turtles with less energy. Congdon and Gibbons (1985) suggested that residual yolk mass may be an important fitness component in hatchlings that overwinter in the nest chamber. Eggs and hatchlings of a species (*Chrysemys picta*) that overwinters in the nest contained higher energetic stores than two species (*Chelydra serpentina* and *Emydoidea blandingii*) that immediately emerge from the nest (Congdon et al. 1983b; Rowe et al. 1995). However, Nagle et al. (1998) found similar lipid proportions among hatchlings from species that overwinter (*Kinosteron subrubrum*) and species with immediate emergence (*Sternotherus odoratus*). Interestingly, eggs from *K. subrubrum* contained higher lipid proportions than *S. odoratus*, indicating that embryonic developmental patterns influence the amount of energy reserves available to hatchlings (Nagle et al. 1998).

Recently, lipid analysis in the diamondback terrapin, *Malaclemys terrapin*, indicated smaller eggs deposited in northern populations that overwinter contained less lipid stores than larger eggs deposited in southern populations with immediate emergence (Allman, Chapter 2). Furthermore, hatchlings from northern populations produced throughout a range of incubation temperatures contained lower energy stores and had lower resting metabolic rates than larger hatchlings from southern populations (Allman, Chapter 3; Chapter 4). These data suggest higher energy stores are important for hatchlings with immediate emergence (Allman, Chapter 4), as has been demonstrated in the loggerhead sea turtle, *Caretta caretta*, (Kraemer and Bennett, 1981) and the snapping turtle, *C. serpentina* (Wilhoft, 1986).

Regardless of the emergence pattern or developmental conditions, residual yolk seems to be an important component of a hatchling's energy budget. In the lizard, *Calotes versicolor*, removal of a large portion of egg yolk resulted in smaller hatchlings with energy stores similar to the control eggs (Radder et al. 2004). This indicates the embryonic lizards sacrificed body size to ensure the presence of residual yolk for the hatchling. Residual yolk in oviparous reptiles is used for dispersal away from the nest (Kramer and Bennett, 1981), maintenance metabolism (Congdon and Gibbons, 1990; Nagle et al. 1998), and even growth during the neonatal period when feeding is likely to be inefficient (Tucker et al. 1998). For this reason, the rate at which individuals utilize the residual yolk may be critical to hatchling survival during this period.

The goal of this study is to test the assumption of the energetic constraint hypothesis that hatchlings utilize residual energy stores at a higher rate in warmer climates. Specifically, I measure whole body energy stores in hatchlings from three

populations raised in cold and hot environmental conditions. I predict that hatchlings from all populations in the hot environment will utilize residual energy stores faster than hatchlings in the cold environment.

Materials and Methods

During the 2005 nesting season, I collected diamondback terrapin, *Malaclemys terrapin*, eggs from populations in South Carolina (32°47'N, 79°56'W), Maryland (38°27'N, 76°39'W), and Rhode Island (41°74N, 71°31'W). Freshly deposited eggs were collected through nesting surveys (Maryland and Rhode Island) and oxytocin injections of captured gravid females (South Carolina; Ewert and Legler, 1978). The eggs were packed in moist sand and stored below 20° C for transport to Ohio University.

I incubated all eggs in a Percival Model 30-BLL incubator set at a constant temperature of 29.5° C. The eggs were randomly placed in individual plastic compartments containing a vermiculite: water (1:2 ratio) substrate. The plastic containers were autoclaved and acid-washed to minimize any leaching of plastic material into the substrate. Additionally, the vermiculite was autoclaved to minimize bacterial growth during incubation. Water was added throughout the incubation period as needed to maintain equal substrate moisture levels in all containers. I rotated the egg containers twice each week to minimize the potential effects of small temperature gradients within the incubator.

Upon hatching (RI: n=30, MD: n=49, SC: n=38)), the mass (to the nearest 0.1g) was measured on all hatchlings, and then a sub-sample (RI: n=4, MD: n=4, SC: n=4) was immediately sacrificed for lipid analysis. The remaining hatchlings from each population were randomly assigned one of two growth treatments representing either a cold or hot

climate condition. The cold growth room mimicked the light cycle and ten-year mean water temperature in Rhode Island (NODC/NOAA, 2004). The hot growth room mimicked the light cycle and ten-year mean water temperature in South Carolina (NODC/NOAA, 2004). I adjusted the light cycle and water temperature every 15 days. Each turtle was raised in an acid-washed plastic housing unit (35 cm L X 19 cm W X 11 cm D) containing water at 10 ppt salinity. Hatchlings were fasted to ensure that energy utilized for maintenance metabolism, and any growth, was from residual yolk energy stores only. The containers were rotated twice per week to account for small variations in temperature within the growth rooms. I measured hatchling mass to the nearest 0.1 mm every 30 days. I used a ANCOVA with hatchling dry mass as the dependent variable to determine differences in mass change among population source and growth treatment room. Wet egg mass was used as a covariate to account for differences in initial egg size.

At hatching and at month one, two, four, and six, a sub-set of hatchlings from each population and growth treatment were sacrificed for lipid analysis. After sacrificing, hatchlings were dried to a constant mass at 60° C and then ground to an even particle size. The sample was then transferred to a new pre-weighed Whatman cellulose thimble (33 X 80 mm) and then placed into a SoxTech HT2 1045 extraction unit. Petroleum ether was refluxed through the samples for 90 minutes at 100° C. I then rinsed the samples in petroleum ether for 45 minutes before collecting the solvent for 15 minutes. The extracted lipids were dried in a drying oven at 60° C for 60 minutes before weighing to the nearest 0.0001 g. The efficiency of this procedure has been previously reported (Roosenburg and Dennis, 2005; Allman, Chapter 3). An ANCOVA was used to determine the differences in lipid mass among hatchlings from different populations and

different growth treatments at different ages. Hatchling lean dry mass was used as a covariate to account for differences in body size.

Results

The mean hatchling mass varied among populations (ANCOVA, $F_{2, 118}$=24.1, P<0.0001), and did not vary with sex (ANCOVA, $F_{1, 118}$=0.14, P=0.714). Hatchlings from South Carolina eggs had a mean mass of 8.6 g (±0.10) and hatchings from Rhode Island weighed 7.3 g (±0.12) (Table 5.1). However, the hatchling dry mass did not vary with population (ANCOVA, $F_{2, 12}$=1.29, P=0.332) or sex (ANCOVA, $F_{2, 12}$=0.67, P=0.441), suggesting that hatchling mass differences can be explained by differences in water content or that the sample size was too small to get a difference. The amount of whole body NPL energy stores also varied among populations (ANCOVA, $F_{2, 12}$=11.0, P=0.003). The South Carolina hatchlings contained 0.55 g (± 0.02) of NPL energy stores, which represents 26% of the dry hatchling mass. Hatchlings from the Rhode Island eggs contained 0.45 g (± 0.02) of NPL energy stores, which represent 23% of the dry hatchling mass. These data indicate that larger hatchlings produced from SC eggs contain a larger proportion of NPL energy stores than hatchlings from MD or RI eggs.

The amount of NPL lipid stores utilized by hatchlings during the six-month study varied with population (ANCOVA, $F_{2, 104}$=39.6, P<0.0001) and treatment room (ANCOVA, $F_{2, 104}$=131.1, P<0.0001). Hatchlings from all populations in the cold treatment room contained more NPL energy stores than hatchlings in the hot treatment room (Figure 5.1). The mass of NPL lipid stores decreased over time (ANCOVA, $F_{3, 104}$=68.6, P<0.0001) throughout the six-month period. These data indicate the hatchlings

were exploiting their energy stores and that the rate of utilization was dependent on the growth treatment room.

The hatchling dry mass varied with treatment room (ANCOVA, $F_{21\ 104}$=12.6, *P*=0.001) and population source (ANCOVA, $F_{2,\ 104}$=3.9, *P*=0.041) over time (ANCOVA, $F_{4,104}$=42.9, *P*<0.001) (Figure 5.2). However, there was a three way interaction effect among treatment room, populations source, and time (ANCOVA, $F_{7,104}$=7.9, *P*<0.001).

Discussion

The purpose of this study was to determine if turtle hatchlings utilize NPL energy stores at a higher rate in warmer climate conditions. For all populations, hatchlings in the hot room utilized NPL energy stores faster than hatchlings in the cold room. Additionally, hatchlings in the hot room grew to larger sizes than hatchlings growing in the cold room. These data indicate hatchlings in warmer climates may utilize energy stores at a higher rate for purposes of increased growth as well as the increased maintenance metabolism (Allman, Chapter 4).

Turtles, like many other oviparous organisms, do not provide post-ovulatory parental care to their offspring. As a result, any parental care provided to the offspring is directed through investment of egg yolk in access to what is required to produce a fully developed hatchling (Congdon and Gibbons, 1990). This access yolk represents the parental investment for care (PIC), and is typically greater than 50% of the original mass of the egg yolk (Congdon and Gibbons, 1985; Congdon et al. 1983a; Wilhoft, 1986; Nagle et al. 2003. This residual yolk is utilized by the hatchling to disperse from the nest cavity, growth, and for maintenance metabolism during the time in which the hatchling is existing with a negative energy budget (Kramer and Bennett, 1981; Marlen and Fischer,

1999; Nagle et al., 2003). In a lizard, one-week-old hatchlings receive 146% of their estimated daily energy requirement from catabolizing energy stores within the residual yolk (Troyer, 1983). Additionally, hatchling smooth softshell turtles, *Apalone mutica*, have residual yolk masses that represent 75% of the original yolk mass in the eggs (Nagle et al. 2003). The softshell turtle lives in fast moving streams that are considered a low-resource environment (Fitch and Plummer, 1975). It follows that the hatchling resource environment may influence selection on PIC, and therefore, on NPL levels in eggs (Troyer, 1983; Congdon and Gibbons, 1991; Fischer et al. 1991; Nagle et al. 2003).

Similarly, the hatchling climatic environment may also influence selection on parental investment and egg size. Recently, Allman (Chapter 4) demonstrated that *M. terrapin* hatchlings with larger energy stores received a survivorship and growth advantage in warmer climate conditions. For many reptile species, including the *M. terrapin*, resting metabolic rate increases with increasing temperature (Angilletta, 2001; Litzgus and Hopkins, 2003; Allman, Chapter 4), indicating that warmer temperatures increase the energetic requirement for maintenance metabolism in ectotherms. In the current study, I demonstrate that hatchlings in warmer climates will likely utilize NPL lipid stores at a faster rate than hatchlings in cooler climates.

Many species of turtles exhibit a latitudinal cline in egg size where females in northern populations deposit small eggs compared to the larger eggs produced by females in southern populations (Fitch, 1985; Iverson et al. 1993). Because egg size typically co-varies with yolk size, and thus yolk NPL levels, increased female investment for larger eggs results in larger hatchlings (Cox and Marion, 1978; Ewert, 1979; Rowe et al. 1995; Nagle et al. 2003). Unfortunately, until recently hypotheses used to explain egg size

variation in turtles have focused on female fecundity or hatchling body size without considering hatchling energetics (reviewed in Iverson et al. 1993). However, hatchling energy reserves may influence hatchling quality by providing energy for growth and survivorship until hatchlings attain a positive energy budget. Therefore, the energetic demand of the hatchlings may influence selection acting on parental investment, such that females depositing larger eggs with more PIC may be favored in warmer climates where the NPL energy stores are utilized at a faster rate. In this paper, I demonstrate that hatchlings utilize energy stores at a faster rate in warmer climates and there appears to be a growth advantage to having more residual energy stores than hatchlings will less energy.

In summary, I (Allman, Chapter 2) have recently demonstrated a variation in egg size among populations of *M. terrapin*, where larger eggs are being produced in southern populations with a cost of clutch size. Additionally, these larger eggs contain a higher proportion of NPL energy stores and produce hatchlings with higher energy stores (Allman, Chapter 2, Chapter 3). In a common-garden experiment I (Allman, Chapter 4) demonstrated that hatchlings with higher energy stores have a survivorship and growth advantage over hatchlings with less PIC. Warmer nest incubation temperatures and water temperatures result in a higher energy demand that results in hatchlings utilizing NPL energy stores at a higher rate than if in cooler climate conditions. Consequently, if egg size is a heritable trait in turtles, the hatchling climate environment may be the ultimate factor influencing egg size in *M. terrapin* and other species that produce larger eggs in warmer climates. To better test this hypothesis, the heritability and plasticity of egg size

need to be determined through experiments with adult female turtles from different populations and different species.

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Table 2.1 Egg size measurements from *Malaclemys terrapin* eggs collected in Rhode Island, Maryland, and South Carolina (N, number of clutches; n, number of eggs; values are means ± 1 standard error).

	N / n	Clutch Size	Clutch Mass (g)	Egg Mass (g)	Egg Length (mm)	Egg Width (mm)	Egg Volume (cc)
Rhode Island	12 / 193	16.1 ± 0.65	135.6 ± 7.1	8.4 ± 0.08	32.9 ± 0.13	20.9 ± 0.08	7.6 ± 0.08
Maryland	14 / 171	12.2 ± 0.42	121.2 ± 4.4	9.9 ± 0.09	34.3 ± 0.13	21.2 ± 0.09	8.1 ± 0.08
South Carolina	17 / 102	6.0 ± 0.38	62.4 ± 4.2	10.4 ± 0.08	36.3 ± 0.12	21.8 ± 0.08	9.1 ± 0.08

Table 2.2. Egg yolk components in *Malaclemys terrapin* eggs collected in Rhode Island, Maryland, and South Carolina (N, number of clutches; n, number of eggs; values are means ± 1 standard error).

	N / n	Wet Yolk Mass (g)	Water Mass (g)	NPL mass (g)	Lean Mass (g)	% Water (wet yolk)	% NPL (wet yolk)	% NPL (dry yolk)
Rhode Island	11 / 22	3.95 ± 0.15	2.19 ± 0.18	0.384 ± 0.02	1.37 ± 0.05	53.2 ± 2.81	9.9 ± 0.76	22.3± 0.86
Maryland	9 / 18	4.19 ± 0.14	2.25 ± 0.16	0.471 ± 0.02	1.53 ± 0.05	53.2 ± 2.74	10.3 ± 0.90	22.5 ± 0.45
South Carolina	15 / 15	4.59 ± 0.16	2.42 ± 0.20	0.668 ± 0.02	1.55 ± 0.05	51.5 ± 2.70	14.8 ± 0.83	31.8 ± 0.64

Table 2.3. Yolk lipid components in *Malaclemys terrapin* eggs collected in Rhode Island, Maryland, and South Carolina (N, number of clutches; n, number of eggs; values are means ± 1 standard error; percentages are of proportions of total lipids).

	n	Triacylglycerol (g)	Triacylglycerol (%)	Cholesterol (g)	Cholesterol (%)	Phospholipids (g)	Phospholipids (%)
Rhode Island	8	0.37 ± 0.04	88.0 ± 1.3	0.0062 ± 0.001	1.5 ± 0.05	0.044 ± 0.04	10.4 ± 1.18
Maryland	7	0.44 ± 0.04	85.4 ± 1.8	0.0083 ± 0.01	1.5 ± 0.11	0.073 ± 0.01	13.0 ± 1.69
South Carolina	8	0.65 ± 0.03	81.9 ± 1.1	0.0120 ± 0.001	1.5 ± 0.13	0.134 ± 0.01	16.6 ± 1.05

Table 3.1. The number of males and females produced from eggs collected from Rhode Island, Maryland, and South Carolina incubated at different temperatures. The proportion of males for each temperature is also given.

	27° C			28.5° C			30° C		
	Males	Females	% Male	Males	Females	% Male	Males	Females	% Male
Rhode Island	50	0	100	33	2	5.7	0	45	0
Maryland	36	0	100	23	7	23.3	0	25	0
South Carolina	35	0	100	15	5	25.0	0	21	0

Table 4.1. Results from an ANCOVA examining the effects of population source and incubation temperature on oxygen consumption in hatchling turtles. The covariate was hatchling mass.

Effect	df	F	P
Population source	2, 203	179.8	<0.0001
Incubation temperature	2, 203	187.7	<0.0001
Population source*Incubation temperature	4, 203	182.4	<0.0001

Table 4.2. Results from a repeated measures ANCOVA examining the effects of population source, growth room, sex, and incubation temperature on hatchling mass and change in mass for a six-month period. The covariate was egg mass.

	Hatchling Mass			Change in Mass		
Effect	df	F	P	df	F	P
Population source	2, 215	31.9	<0.0001	2, 215	46.6	<0.0001
Growth	2, 215	139.2	<0.0001	2, 215	166.9	<0.0001
Incubation temperature	2, 215	0.25	0.7781	2, 215	0.49	0.4810
Sex	1, 215	0.56	0.4541	1, 215	0.11	0.7350
Population source*Incubation temperature	4, 215	13.1	<0.0001	4, 215	23.6	<0.0001
Egg Mass	1, 215	124.7	<0.0001	1, 215	4.75	0.0096

Table 5.1. Hatchling mass and non-polar lipid (NPL) mass (± 1 SE) from hatchlings produced from eggs collected from RI, MD, and SC and incubated 29.5° C.

Location	Hatchling Mass (g)	NPL Mass (g)	% NPL Dry Mass	% NPL Wet Mass
Rhode Island	7.3 ± 0.12 n=30	0.55 ± 0.02 n=4	22.5	6.5
Maryland	8.2 ± 0.16 n=49	0.48 ± 0.02 n=4	23.6	6.8
South Carolina	8.6 ± 0.10 n=38	0.45 ± 0.02 n=4	26.0	7.0

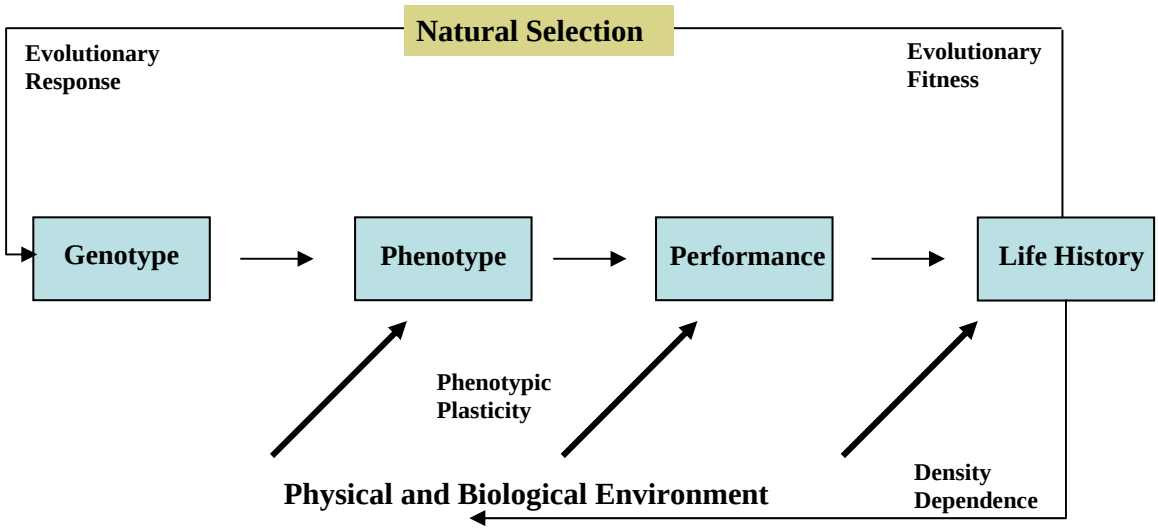

Figure 1.1. Paradigm of life history evolution associated with environmental and evolutionary feedback on phenotypes (modified from Arnold, 1983 and Ricklefs, 2000).

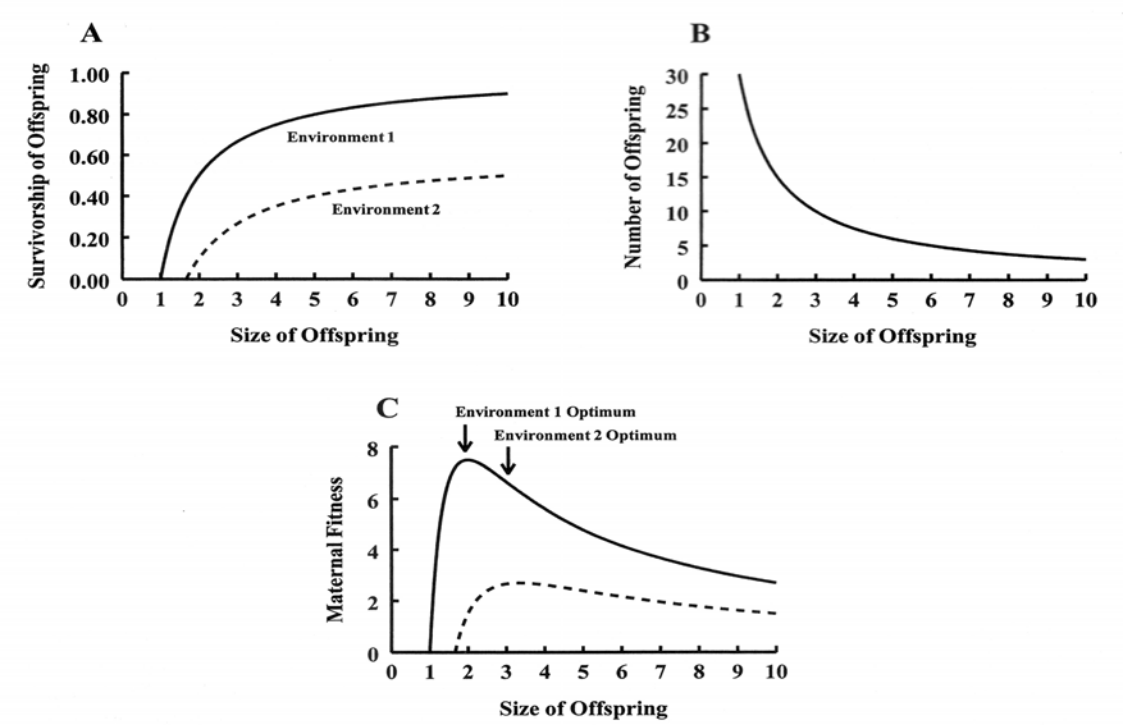

Figure 1.2. Illustration of the Smith-Fretwell model of optimal egg size. (A) Offspring fitness increases with increasing offspring size. (B) There is a tradeoff between offspring size and number. (C) Maternal fitness is maximized by the production of different size offspring in different environments (from Messina and Fox, 2001).

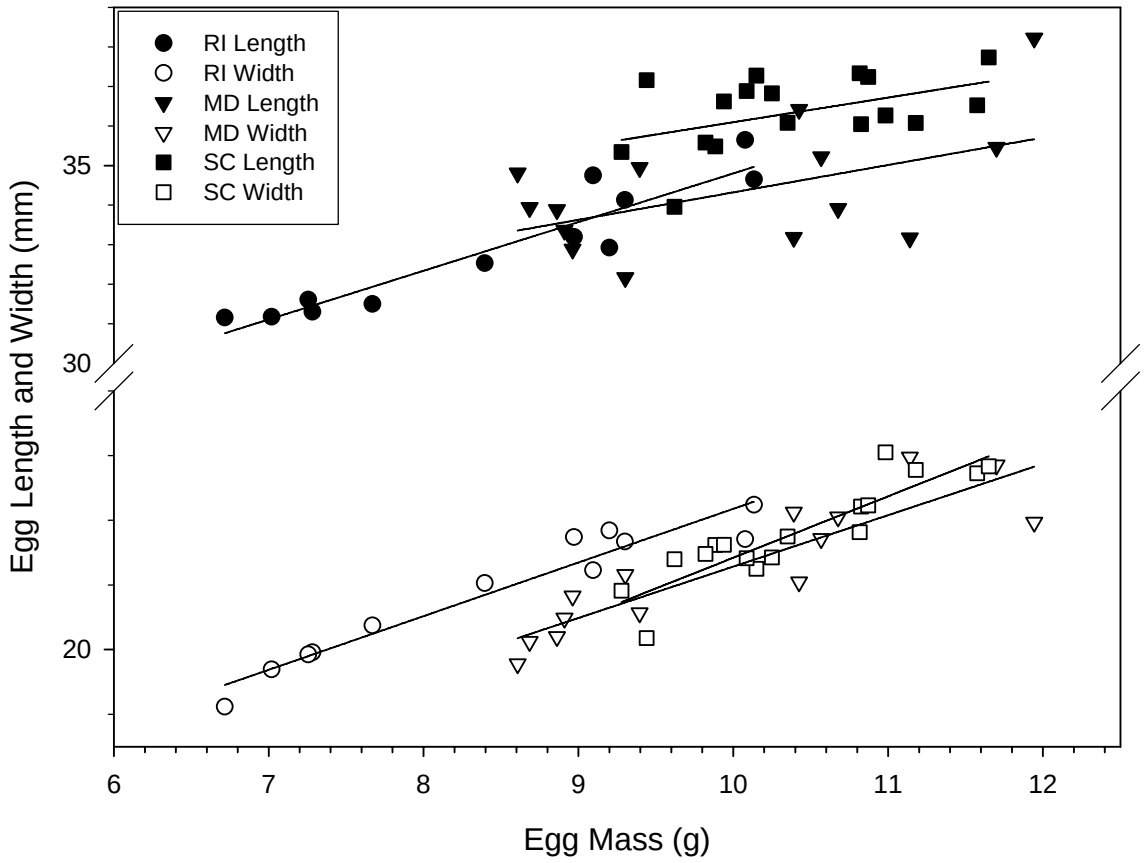

Figure 2.1. The relationship between egg width (open) and egg length (closed) to egg mass in *Malaclemys terrapin* eggs collected from Rhode Island, Maryland, and South Carolina.

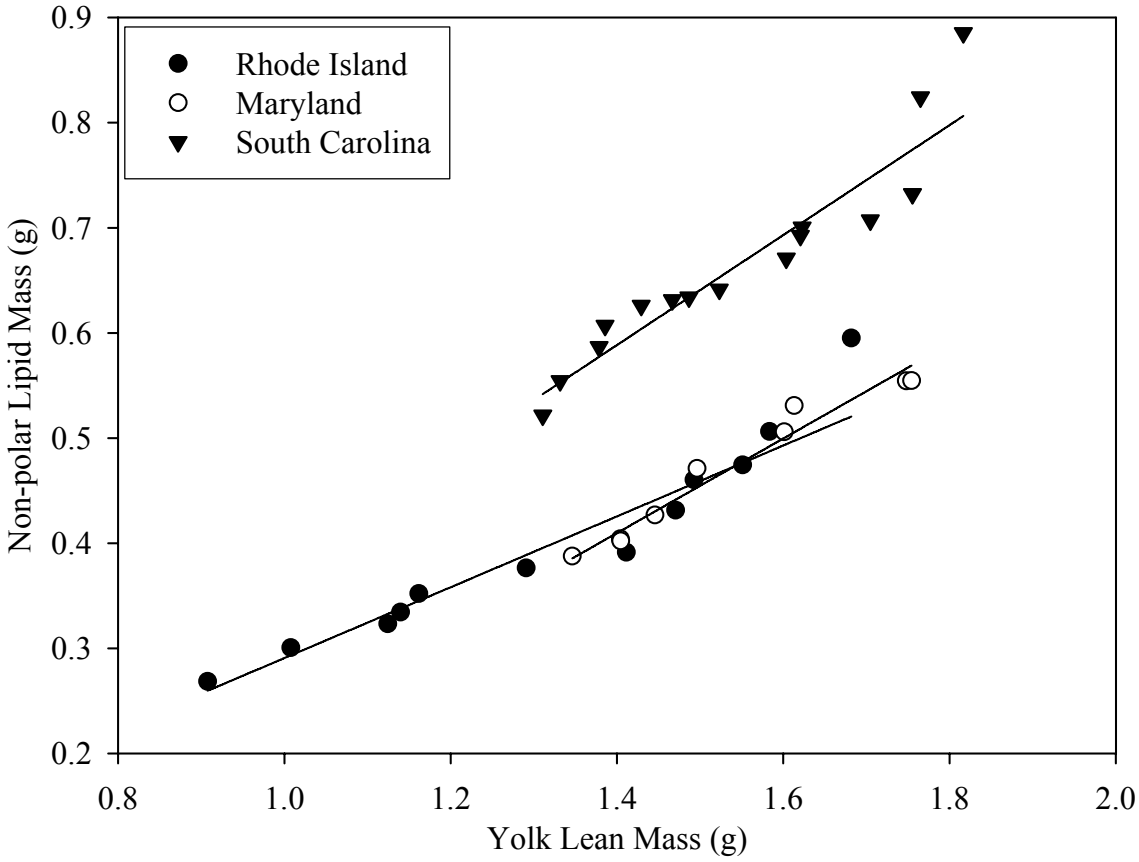

Figure 2.2. The relationship between non-polar lipid (NPL) mass and yolk lean mass in *Malaclemys terrapin* eggs collected from Rhode Island(r^2=0.93, y=0.37x-0.086), Maryland(r^2=0.96, y=0.44x-0.204, and South Carolina (r^2=0.89, y=0.55x-0.178).

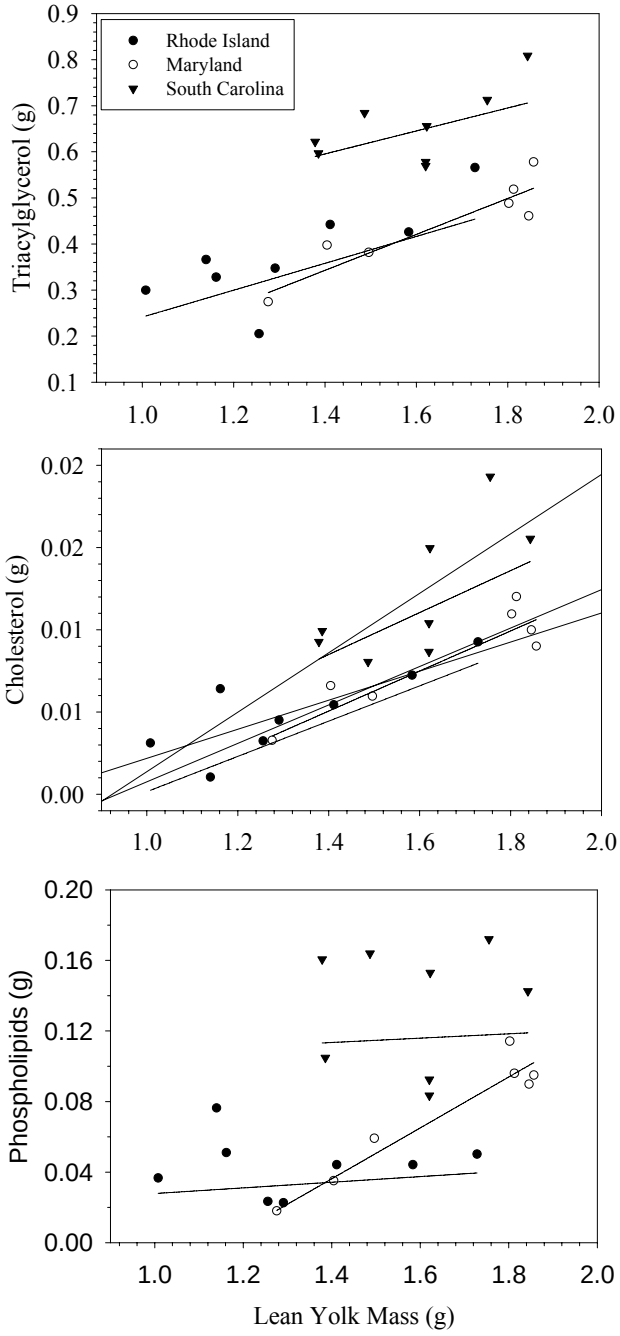

Figure 2.3. Relationships of triacylglycerol, cholesterol, and phospholipids from *Malaclemys terrapin* eggs collected in Rhode Island, Maryland, and South Carolina.

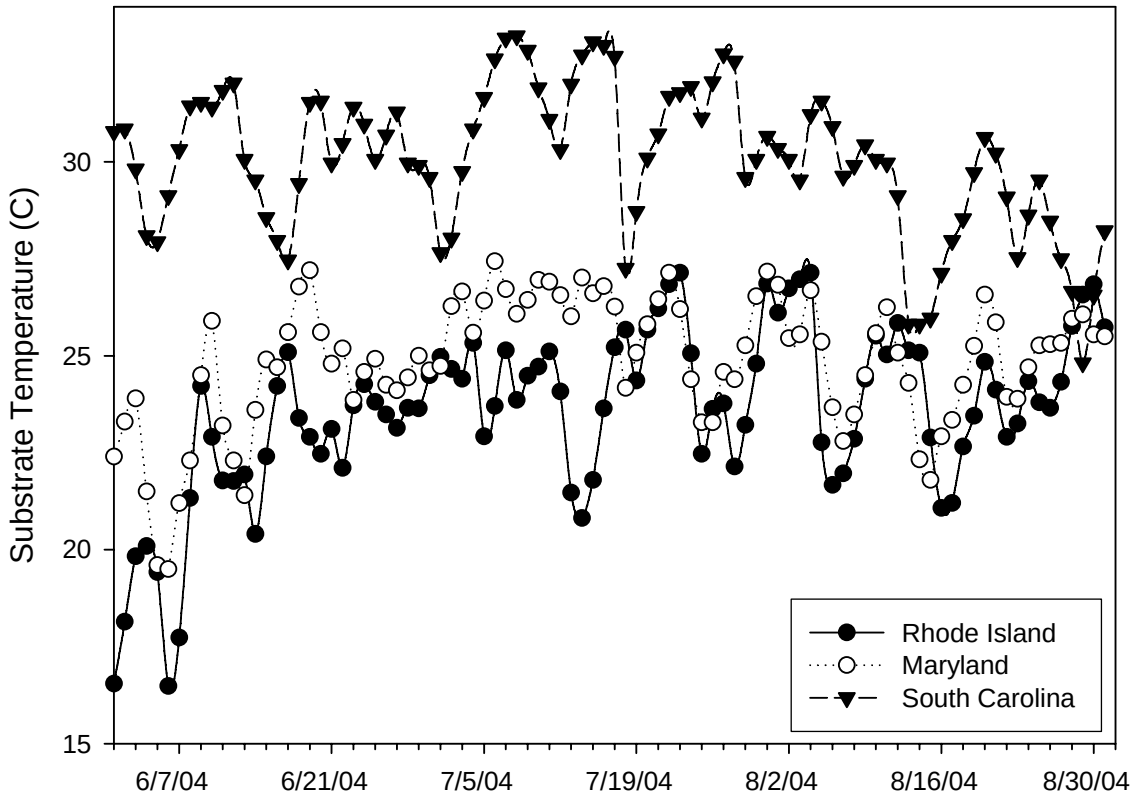

Figure 3.1. Recorded substrate temperature at nest-depth among three *Malaclemys terrapin* nesting sites. Symbols indicate the 24 hour mean temperature for each day from June 1, 2004 to August 31, 2004 in Rhode Island, Maryland, and South Carolina.

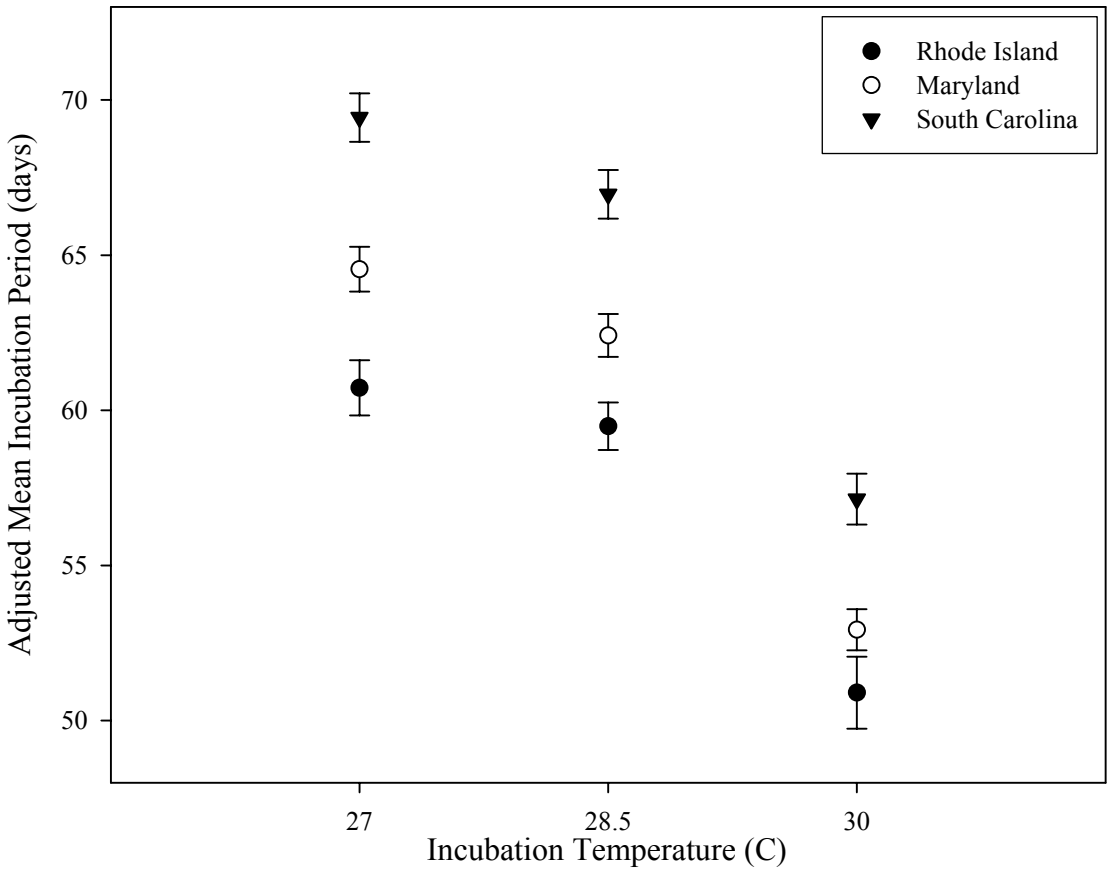

Figure 3.2. Least square mean values of incubation temperature (± 1 SE) for hatchlings deposited from eggs collected in Rhode Island, Maryland, and South Carolina incubated at one of three constant incubation temperatures.

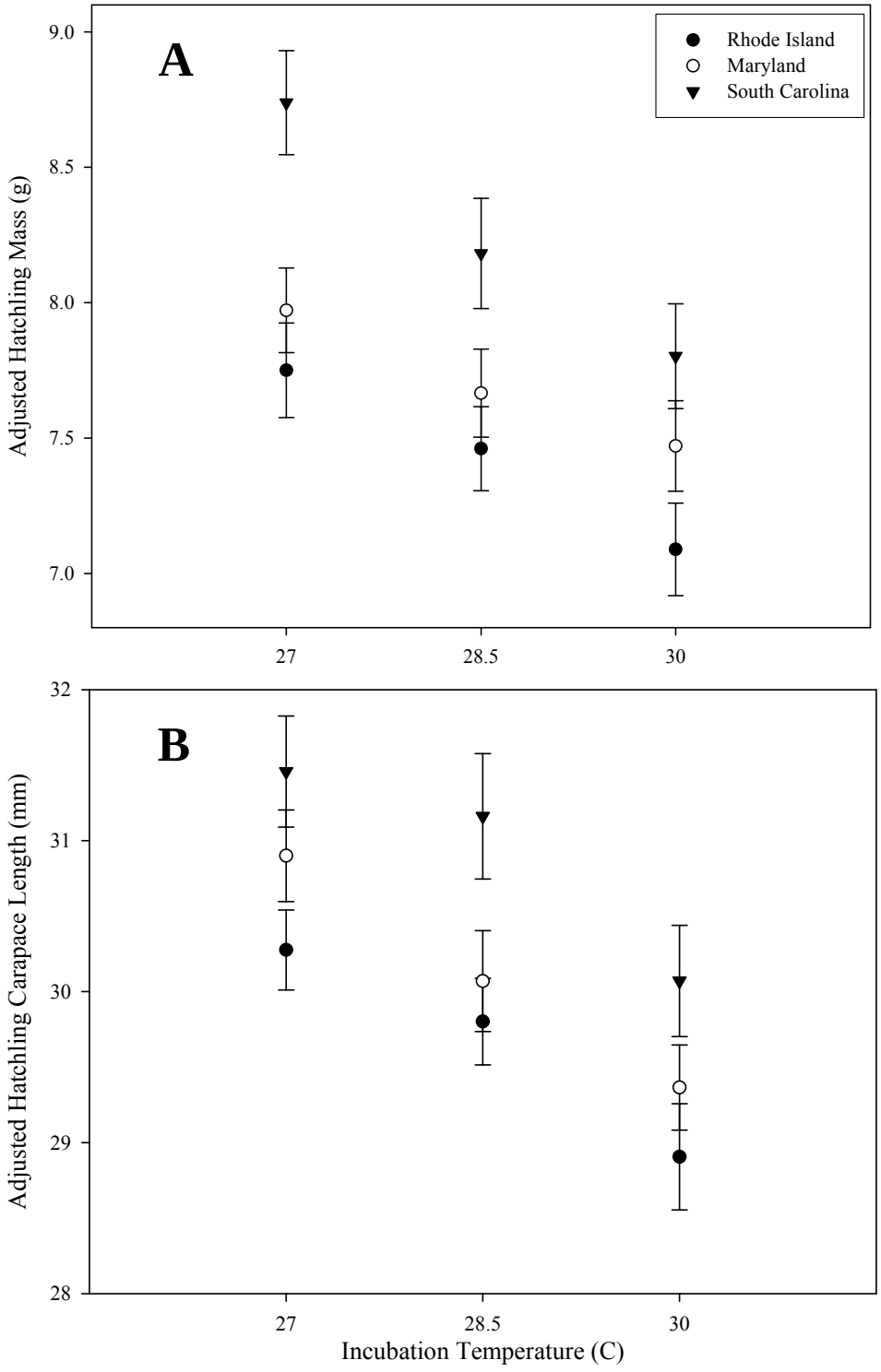

Figure 3.3. Least square mean values of hatchling mass (A) and carapace length (B) (± 1 SE) for hatchlings from eggs collected from three populations and incubated at different temperatures.

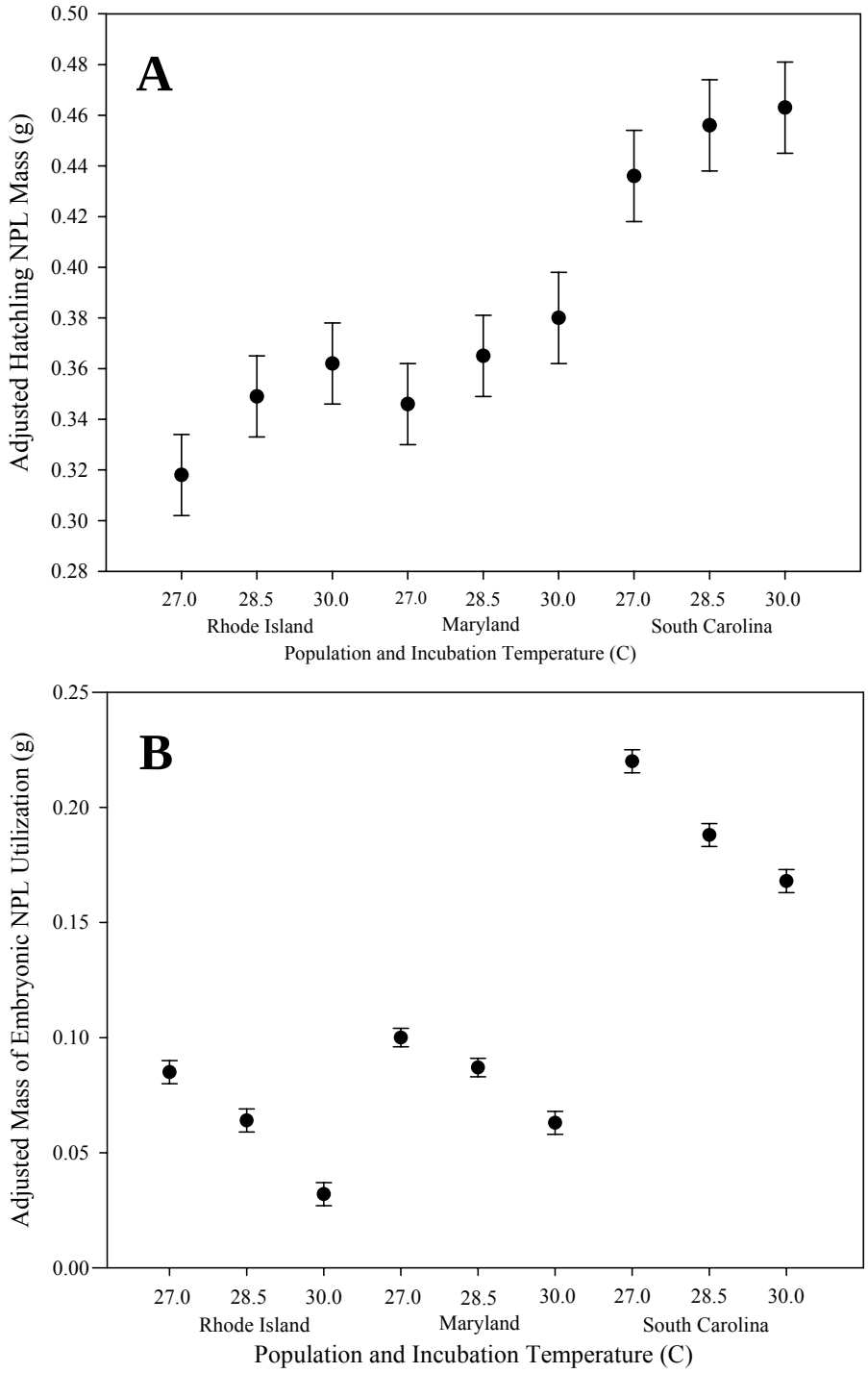

Figure 3.4. Least square mean values of non-polar lipid mass (± 1 SE) in hatchlings (A) from eggs collected in Rhode Island, Maryland, and South Carolina incubated at different temperatures. The adjusted mass of non-polar lipids utilized (± 1 SE) during incubation (B) at each temperature was calculated from the clutch mean non-polar lipid mass (Allman, Chapter 2) and the mass of non-polar lipids upon hatching.

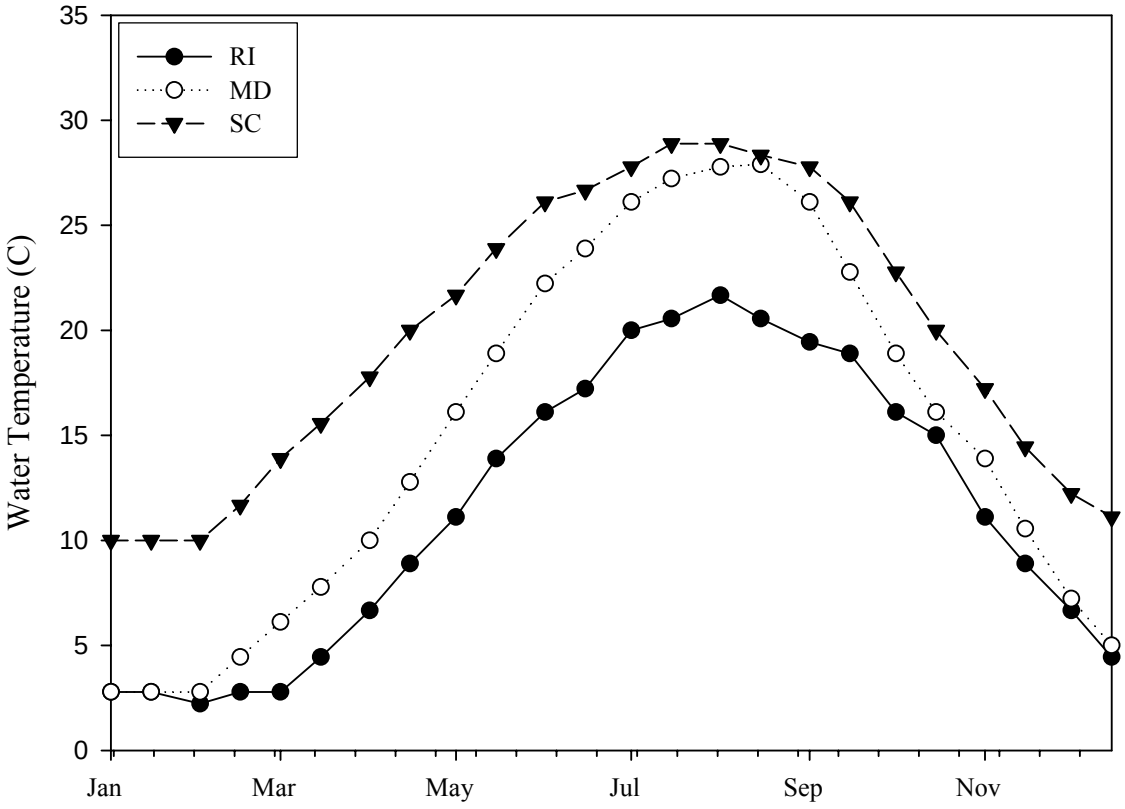

Figure 4.1. Ten-year mean water temperatures recorded from buoys located near the collection sites in Rhode Island, Maryland, and South Carolina. Data were obtained from the National Oceanographic Data Center (NODC/NOAA, 2004).

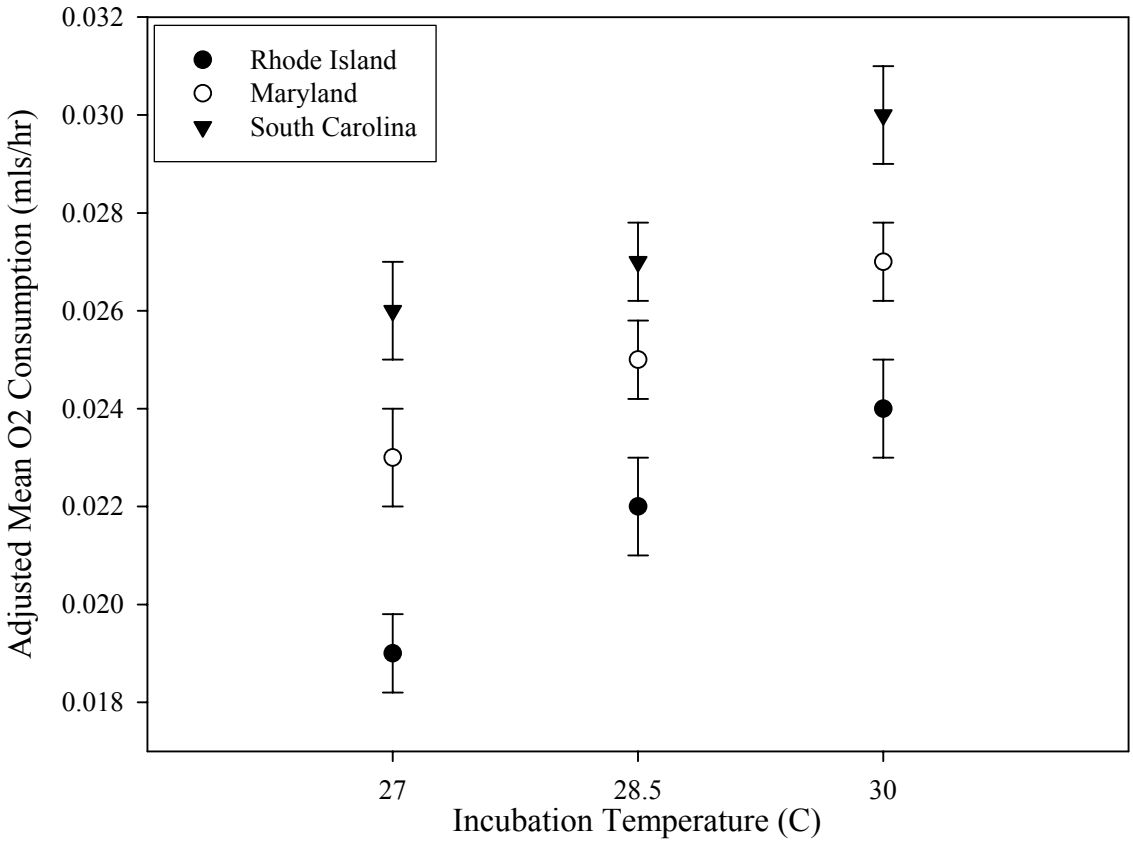

Figure 4.2. Least square mean values of resting metabolic rate measured in neonate turtles from three incubation temperatures. Oxygen consumption was measured through a closed system respirometer. Values are least square means ±1SE.

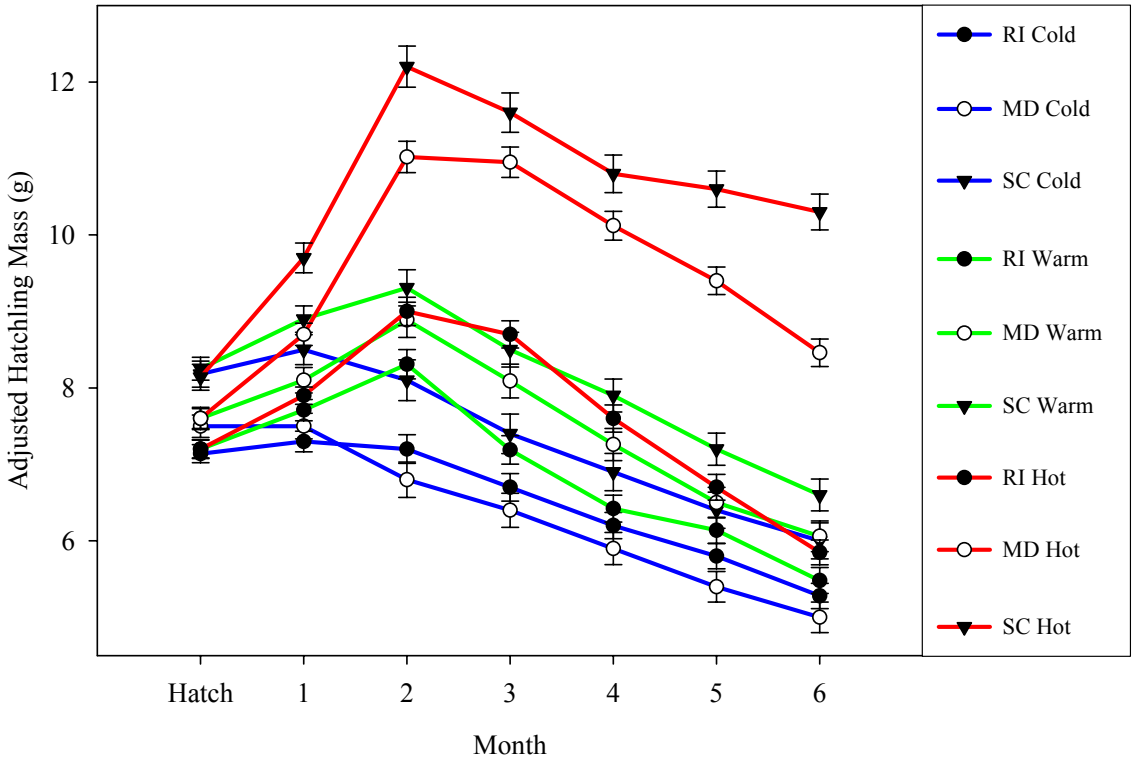

Figure 4.3. Least square mean values of hatchling mass measured for six months in hatchlings from three populations raised in treatment rooms mimicking conditions of the source populations (SC = hot room, MD = warm room, RI = cold room). Values are least square means ±1SE.

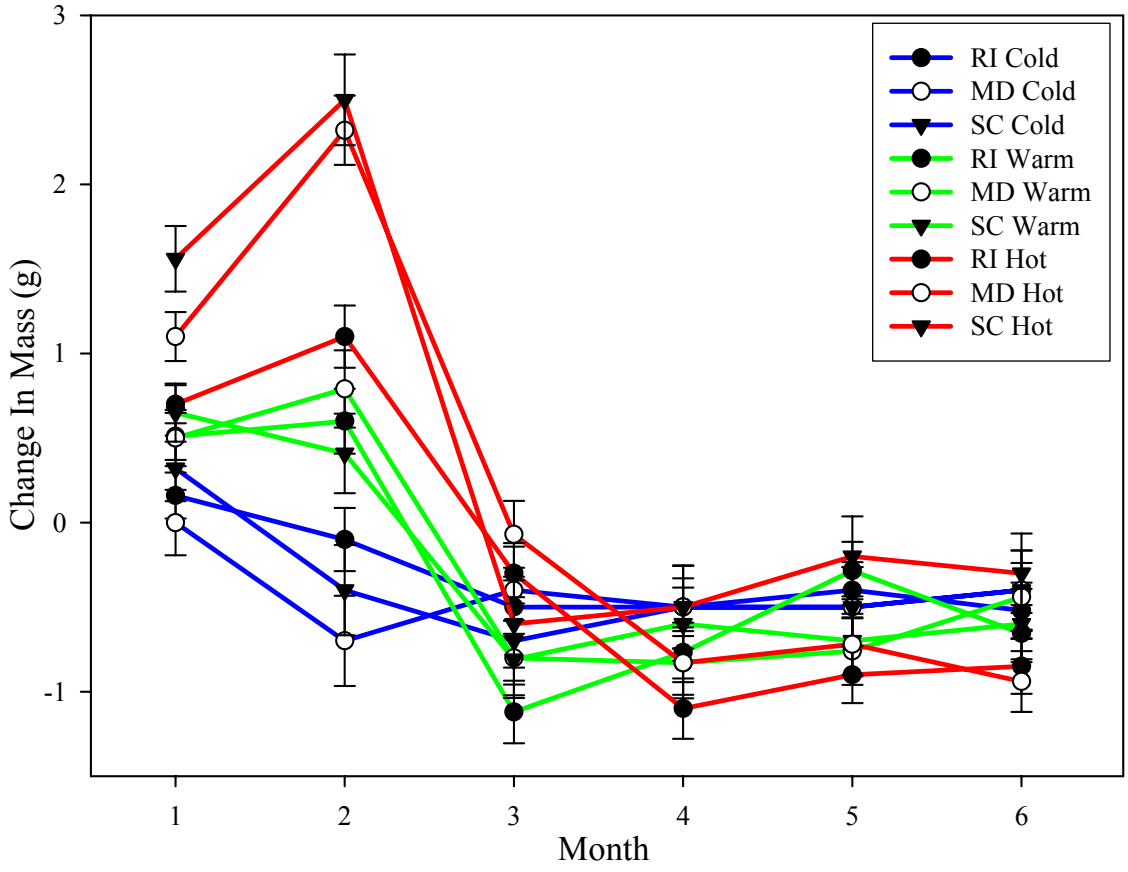

Figure 4.4. Change in mass during each 30-day period for six months in hatchlings from three populations raised in treatment rooms mimicking conditions of the source populations (SC = hot room, MD = warm room, RI = cold room).

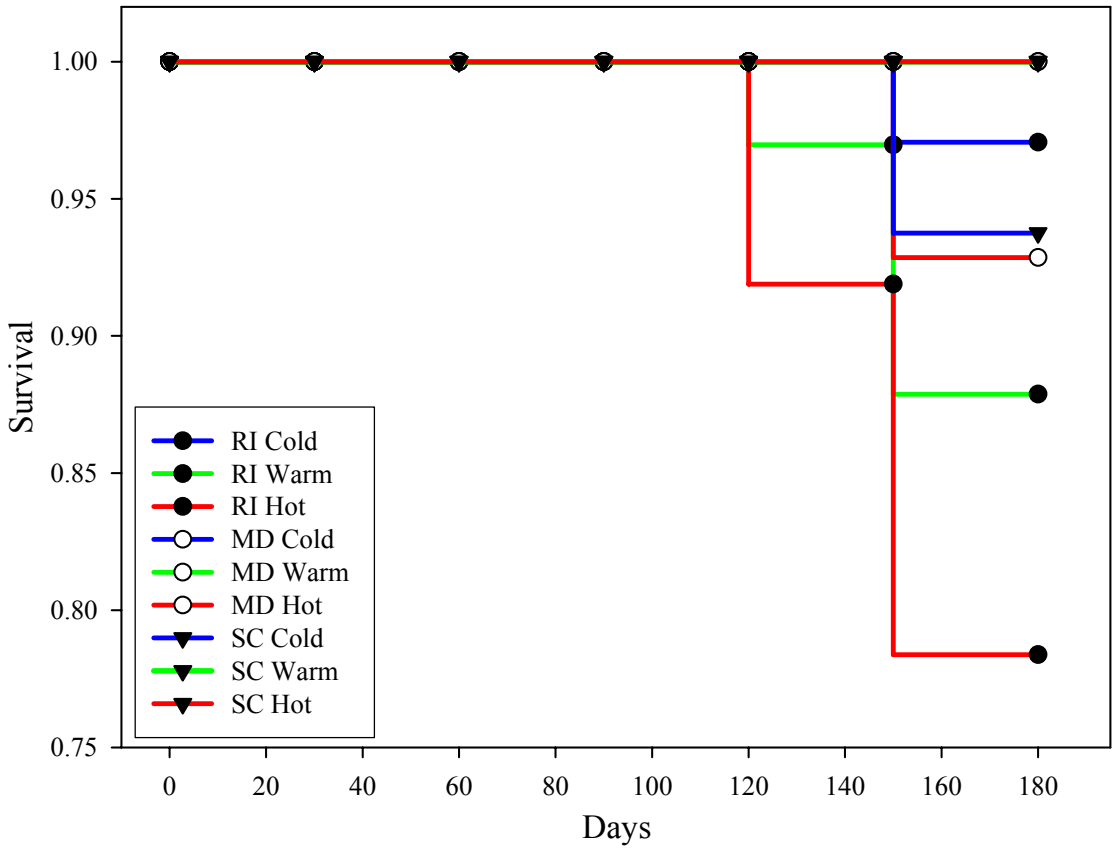

Figure 4.5. Kaplan-Meier survivorship curves for hatchlings from three populations raised in in treatment rooms mimicking conditions of the source populations (SC = hot room, MD = warm room, RI = cold room).

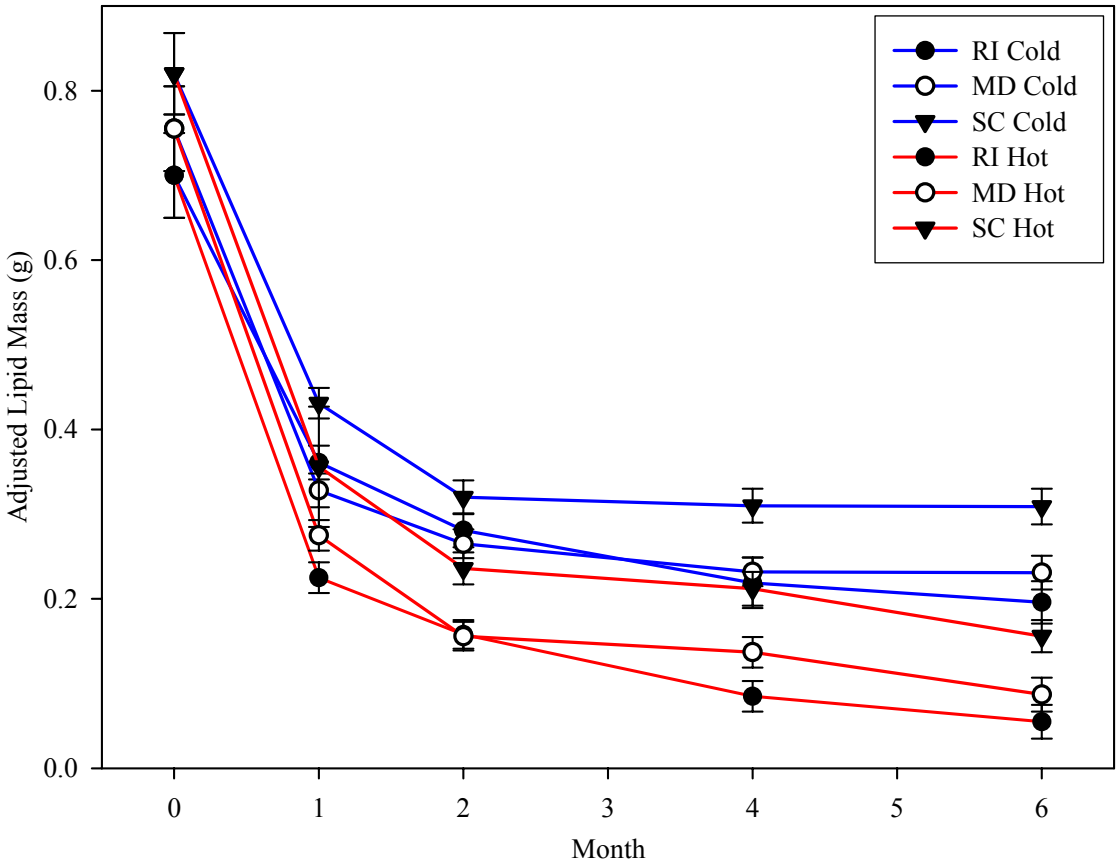

Figure 5.1. Least square mean values of lipid mass for *Malaclemys terrapin* hatchlings from RI, MD, and SC at age 0, 1, 2, 4, and 6 months of age. All hatchlings were fasted for the length of the experiment and raised in a cold or hot growth treatment room. Adjusted lipid mass varied among populations (ANCOVA, $F_{2, 104}$=39.6, P<0.0001) and treatment room (ANCOVA, $F_{2, 104}$=131.1, P<0.0001).

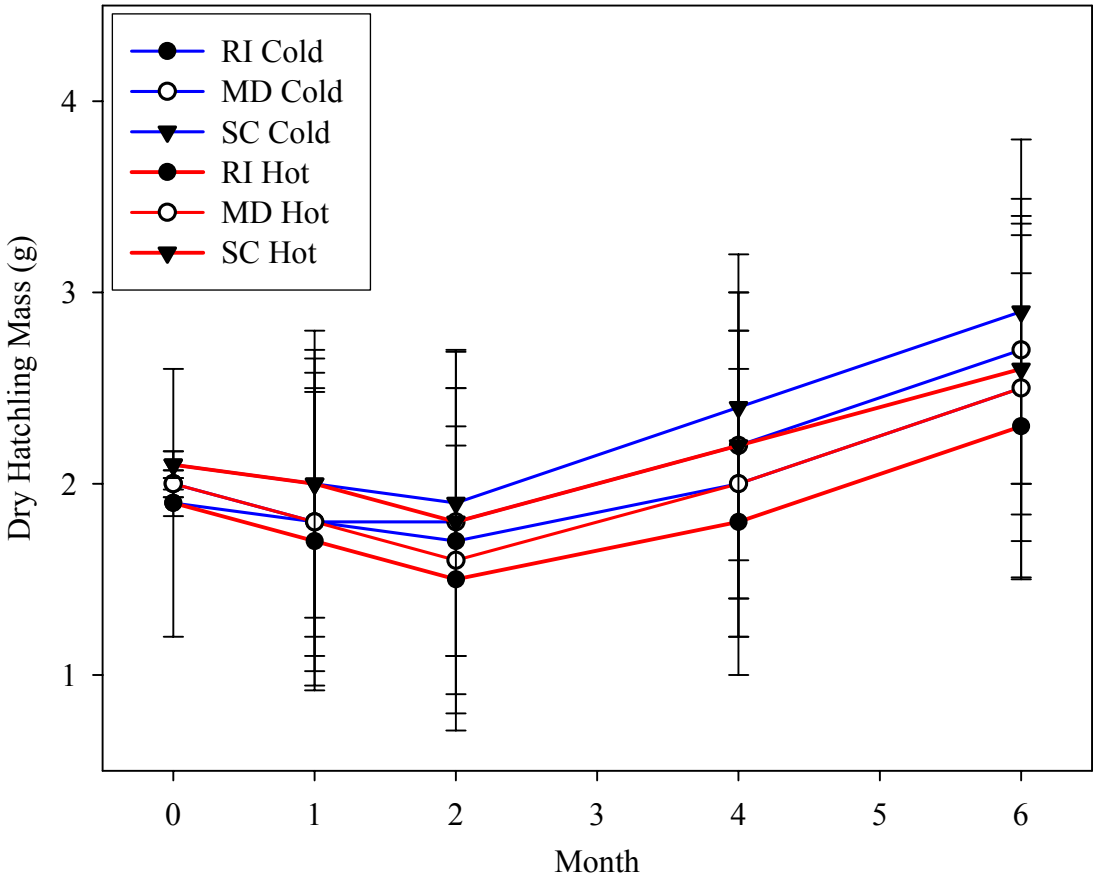

Figure 5.2. *Malaclemys terrapin* dry hatchling masses from three populations raised in a cold or hot growth treatment room. The hatchling dry mass varied with treatment room (ANCOVA, $F_{21\ 104}$=12.6, *P*=0.001) and population source (ANCOVA, $F_{2,\ 104}$=3.9, *P*=0.041) over time (ANCOVA, $F_{4,104}$=42.9, *P*<0.001) (Figure 5.2). However, there was a three way interaction effect among treatment room, populations source, and time (ANCOVA, $F_{7,104}$=7.9, *P*<0.001).