

The University of Southern Mississippi

Metabolic Rate of Gopher Tortoise (*Gopherus polyphemus*) Embryos

by

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## Chapter 1: The Problem

The DeSoto National Forest in Mississippi is home to the largest population of gopher tortoises (*Gopherus polyphemus*) in the western portion of their range (Noel, 2006). The gopher tortoise, the only tortoise found east of the Mississippi River, is a large terrestrial tortoise whose burrows serve as a refuge for many vertebrates (Wilson, Mushinsky, & Fischer, 1997). The western population of tortoises has been declining in recent years, leading to its classification as “threatened” by the federal government and “endangered” by the state of Mississippi (Wilson et al, 1997).

The decline of the western population has been associated with low recruitment, which is unaided by low hatching success (Epperson and Heise, 2003; Noel, 2006). In Florida, hatching success in naturally incubated nests was 80.6% (Butler and Hull, 1996), whereas the hatching success rates for gopher tortoises in Mississippi was found to be 28% by Epperson and Heise (2003) and 17.1% by Noel (2006). In contrast a study by Noel (2006) documented a hatching success rate of 58.8% in an incubator. The 41.7% discrepancy between hatching success in the wild and in the incubator, along with the presence of late-term embryos in many of the unhatched eggs, suggests that some aspect of the natural nest environment is contributing to the failure of eggs that are otherwise capable of hatching successfully (Noel, 2006). The clay content of the soil was correlated with the hatching success among natural nests, which Noel (2006) suggested might be due to the detrimental effects of clay on gas exchange. Limited gas exchange is known to increase egg mortality (Ackerman, 1981). The purpose of this study is to quantify the oxygen needs of gopher tortoise embryos at different temperatures and stages of development. These data will be compared to other data measuring the amount of oxygen available in natural nests, to infer whether and/or how frequently embryonic gas exchange needs

exceed that available in the nests.

Interstitial oxygen levels are impacted by the substrate of the nest. In tortoise nests, gas exchange between the atmosphere and the egg is impeded by the substrate, which typically serves to increase levels of carbon dioxide and decrease levels of oxygen (Deeming & Thompson, 1991). Diffusion is the main mode of gas exchange between the substrate and the atmosphere, as well as between tortoise eggs and the substrate (Prange & Ackerman, 1974). Female turtles have evolved to cope with the nest microhabitat by actively selecting microhabitat factors such as substrate type to increase offspring fitness (Wilson, 1998; Spencer & Thompson, 2002).

Because of the large variation between the nest microhabitat in the eastern range and the western range, new methods of nest searching were developed for the western population (Epperson & Heise, 2003). Gopher tortoises tend to prefer open sandy soils for their nests in Georgia and Florida (Butler & Hull, 1996; Landers, Garner, &McRae, 1980) but are forced to use higher clay soils in the west. As the oxygen demands of the embryo increases after the first third of its development (Leshem, Ar, & Ackerman, 1991), the tortoises might become more vulnerable to drowning or suffocating due to low oxygen levels brought on by heavy rains (Noel, 2006). Clay retains more water than sand, and oxygen diffuses more slowly through water than through air (Deeming & Thompson, 1991). In addition to moisture content of the soil, the presence of decaying embryos in the nest might affect oxygen demand in nests, by consuming oxygen that would otherwise be available for embryonic metabolism.

This research will answer two basic questions: 1.) what is the oxygen consumption of *Gopherus polyphemus* embryos at different temperatures across different stages of development, and 2.) what is the impact of the dead eggs on the level of oxygen in the nest? Eggs are naturally

subjected to a variety of temperatures during incubation, with temperatures fluctuating with daily sun movement and with the month of incubation. Metabolic rates in reptiles are correlated with temperature (Vleck and Hoyt, 1991), so it is necessary to know the metabolic rates of the embryos at several different temperatures if conclusions about the supply of oxygen are to be made. The oxygen consumption rates of decomposing eggs will also be measured to determine if decomposing eggs become oxygen sinks. The low hatching success rate makes dead embryos in the nests common. The eggs undergo anaerobic decomposition, as is detected by the odor of the eggs as they are cracked open for examination (C. Qualls, pers. comm., May 15, 2006), suggesting that oxygen is limited within these eggs. If the internal environment of the decomposing eggs is anaerobic, it is possible that this reflects hypoxic or anoxic conditions in the nest that may negatively affect nearby embryos.

## Chapter 2: Literature Review

The gopher tortoise (*Gopherus polyphemus*) has been declining in numbers in both the eastern and western portion of its range. The western population includes the Mississippi population of gopher tortoises, which have been classified as endangered by the state of Mississippi and threatened by the federal government (Wilson, Mushinsky, and Fischer, 1997). Studies on hatching success and hatchling mortality (Epperson and Heise, 2003), as well as the intrinsic and extrinsic factors affecting hatching success (Noel, 2006) have been conducted in the DeSoto National Forest in Mississippi. The low hatching success rate is partly due to intrinsic factors such as genetics and partly due to extrinsic factors such as nest environment (Noel, 2006). While no statistically significant relationships between substrate and hatching success was found in her study, Noel (2006) postulated that high clay soil, when paired with summer

thunderstorms, may cause the hatchlings to drown or suffocate late in development.

Studies examining growth rates, energy consumption, and development are often conducted by measuring metabolic rates, which can be confounded by factors such as temperature, physical activity, and environmental stressors (Vleck & Hoyt, 1991).

Developmental studies often utilize eggs to bypass confounding factors, since eggs provide a relatively closed system that is completely independent of the mother after oviposition (Vleck & Hoyt, 1991). Studies on the ontogeny of turtle embryos are often conducted by coupling metabolic rate measurements with measurements of dry or wet embryonic weights (Ackerman, 1981; Lynn & von Brand, 1945).

The metabolic rate of an organism can be monitored by measuring three variables: oxygen consumption, carbon dioxide production, or heat production (Vleck & Hoyt, 1991). Researchers examining the metabolic rates of turtle embryos conventionally utilize the volume of oxygen consumed by the embryo (Ackerman, 1981b; Leshem, Ar, & Thompson 1993; Lynn & von Brand, 1945; Peterson & Kruegl, 2005; Thompson, 1993). The oxygen consumption patterns of several members of the Testudinia clade, including *Chelydra serpentina serpentina*, *Kinosternon subrurum subrurum*, and *Terrapene carolina truinguis* (Lynn & von Brand, 1945), *Chelonia* and *Caretta* (Ackerman, 1981b), *Trionyx triunguis* (Leshem, Ar, and Ackerman, 1993), *Dermochelys coriacea* (Thompson, 1993), and *Chrysemys picta picta* (Peterson & Kruegl, 2005), have been measured. The pattern of oxygen consumption of gopher tortoise embryos, however, is unknown.

The effect of environmental factors on the metabolism of tortoise embryos depends on the structure of the egg shell (Gettinger, Paukstis, & Gutzke, 1984). Tortoise eggs tend to be symmetrical, resulting in either spherical or ellipsoid eggs with non-tapered ends (Iverson &

Ewert, 1991). Tortoise eggs have two possible egg shell types: (1) parchment shells, which indent easily and expand through development with the intake of water and (2) rigid shells (Pough, Janice, & Heiser, 2005). The egg shells of turtles are often calcified with a calcium carbonate substance called argonite, and the rigidity of the egg is determined by the organization and thickness of the calcareous layer (Packard & DeMarco, 1991). The eggs of *Gopherus polyphemus* fall in the category of calcareous, rigid-shelled eggs (Wilson, Mushinsky, & Fischer, 1997), with occasional thin, brittle-shelled eggs being recorded in Mississippi (Noel, 2006).

Water can play a significant role in the development and hatching success of turtle eggs (Cagle, Packard, Miller, & Packard, 1993; Spotila, Zimmerman, Binckley, Grumbles, Rostal, List, Beyer, Phillips, & Kemp, 1994). Water potential in nests is a better predictor of increased incubation period and hatchling mass than temperature alone, indicating that the hydric environment has a greater influence on embryonic metabolism than does temperature (Cagle, Packard, Miller, & Packard, 1993). In a study controlling for genetic variation in hatchling size and hatching success, Cagle, Packard, Miller and Packard (1993) found that eggs in cool, moist nests experienced a longer incubation period and produce larger hatchlings. The influence of water on the metabolic rate of the egg was also influenced by the species of egg and the egg structure. The parchment shelled eggs of *Chelydra serpentina serpentina* consumed significantly more oxygen when incubated on wet substrates than did eggs incubated on dry substrates (Gettinger, Paukstis, & Gutzke, 1984). The rigid calcareous eggs of *Trionyx spiniferus*, the soft-shelled turtle, exhibited no differences in oxygen consumption when incubated on wet or dry mediums (Gettinger, Paukstis, & Gutzke, 1984). Unlike *T. spiniferus*, desert tortoise (*Gopherus agassizi*) eggs were affected by moisture content. In *G. agassizi* soil moisture contents higher than 4% caused mortality at low temperatures (Spotila, Zimmerman, Binckley, Grumbles,

Rostal, List, Beyer, Phillips, & Kemp, 1994). The rigid shelled eggs absorb moisture faster than it could evaporate (Spotila et al, 1994). Because it evolved in a more moist environment, the eggs of *Gopherus polyphemus* are expected to be slightly more tolerant to moist conditions than its close relative, *Gopherus agassizi*.

Because a 10°C increase in temperature can potentially double or triple reaction rates, temperature is an important factor when determining metabolic rates (Pough, Janice, & Heiser, 2005). Reptile eggs, because they are generally left untended after oviposition, are exposed to a wider range of temperatures than avian eggs (Hoyt & Vleck, 1991). Temperatures in nests vary with diurnal and seasonal fluctuations, as well as with microhabitat variables such as the amount of shading or cavity depth (Leshem, Ar, & Ackerman, 1991; Thompson, 1988). Temperatures experienced by eggs in the bottom of *Emydura macquarrii* nests were cooler than those near the top for 75% of the day (Thompson, 1988). Temperature variation due to diurnal variation also occurred in gopher tortoise nests (Demuth, 2001; Noel, 2006). Gopher tortoise eggs in Mississippi were exposed to average daily temperature variations of 3.1°C to 7.9°C (Noel, 2006). Temperature is also important in tortoises since several species exhibit temperature-dependent sex-determination. In gopher tortoises, temperatures of approximately 29.3°C produce an equivalent number of male and female offspring (Demuth, 2001). Temperatures above this pivotal temperature produce females, whereas lower temperatures produce males (Demuth, 2001).

Because of the possible impact of temperature on the survival of the embryos in the nest, numerous studies have been conducted examining the relationship between oxygen consumption and temperature (Leshem, Ar, & Ackerman, 1991; Thompson, 1988). The correlation of shorter incubation periods with higher temperatures is clearly evident. Thompson (1988) found eggs

deposited later in the season typically had shorter incubation periods than did those oviposited earlier in the season. Leshem, Ar, and Ackerman (1991) found that the incubation time of *Trionyx triunguis* eggs at higher temperatures was significantly shorter than those incubated at lower temperatures. The extreme low temperature of 24°C in the study resulted in zero hatching success, with the embryos dying late in development (Leshem, Ar, & Ackerman, 1991). Interestingly, the hatchling masses and the total volume of oxygen consumed during incubation of the three temperature groups did not differ significantly, suggesting that the eggs incubated in colder conditions sustained a lower metabolic rate for a greater period of time than eggs incubated at higher temperatures (Leshem et al, 1991). The pattern of oxygen consumption by eggs incubated at higher temperatures had higher peaks, indicating that more oxygen was needed for the increased metabolic rate of eggs exposed to higher temperatures (Leshem et al, 1991). Because the oxygen consumption is affected by environmental temperatures, it is possible that eggs in a natural nest might not be able to obtain enough oxygen to sustain their developmental rate. For gopher tortoise eggs incubated in the field at Camp Shelby, the average daily temperature fluctuation was between an average daily minimum of 24.3°C and an average daily maximum of 32.1°C, with no detrimental affects on hatching success (Noel, 2006).

Development also impacts the level of oxygen consumption of the embryos. Tortoises, like all non-amphibian tetrapods, have an amniotic egg (Pough, Janice, & Heiser, 2005). The amniotic egg consists of four extra-embryonic membranes. These are the amnion, which provides protection from mechanical movement and serves as a storage reservoir for protein and water, the yolk sac, which is highly vascularized so nutrients can be transported from it to the embryo, the allantois, which serves for both nitrogenous waste storage and respiration in later development, and the chorion, which surrounds the entire contents of the egg (Pough, Janice, &

Heiser, 2005). Tortoise embryos are oviposited in the gastrula stage of development, compared to the blastula stage for embryos in bird eggs (Ewert, 1979). The embryonic vitelline membrane, also known as the embryonic disk, attaches to the upper inner shell membrane some time between a few hours and three days after oviposition, forming an opaque circular region at the top of the shell (Ewert, 1979). The opaque region, which contains less water than the translucent region of the egg, continues to grow until the entire egg is covered (Chan, 1989; Thompson, 1989). Embryos obtain nutrition from the yolk sac within the egg for the duration of development. Many turtles complete development with a large residual supply of yolk (Ewert, 1979). Because residual yolk represents unconsumed energy provided by the mother, the differentiation between dry weights and wet weights are important in studies concerning the growth of embryos (Ackerman, 1981a,b; Lynn & von Brand, 1945). Embryonic development is concluded once the yolk sac has been completely internalized and the blood has been taken out of the chorio-allantois membrane (Ewert, 1991).

The oxygen consumption pattern of many turtle embryos across development is either sigmoidal (Lynn & von Brand, 1945; Thompson, 1993) or peaked (Gettinger, Paukstis, & Gutzke, 1984; Leshem Ar, & Ackerman, 1991; Peterson & Kruegl, 2005). Ackerman (1981b) found the oxygen consumption of *Chelonia mydas* and *Caretta caretta* embryos increased throughout the incubation period. However, when regression lines were fit to the data, the exponential decay model, which produces a sigmoidal pattern, fit the data better than a purely exponential growth model (Ackerman, 1981b). In this way, the pattern of oxygen consumption does not differ from the other studies. Other turtles with a sigmoidal pattern are *Chelydra serpentina serpentina*, *Chrysemy picta picta*, *Kinosternum subrubrum subrubrum*, and *Terrapene carolina triunguis* (Lynn & von Brand, 1945). The pattern of oxygen consumption for *T.*

*carolina triunguis* is not conclusively sigmoidal rather than peaked, however, and the study ended when the *T. carolina triunguis* embryos still had an estimated ten days of development (Lynn & von Brand, 1945). Later studies on *C. serpentina* revealed a peaked pattern of oxygen consumption as opposed to the sigmoid pattern found by Lynn and von Brand (Gettinger, Paukstis, & Gutzke, 1984). The difference in the patterns could be attributed to more precise, automated measuring equipment used in the later experiment. Not all sigmoid patterns can be attributed to out-dated equipment, however. Thompson (1991) also recorded a definite asymptote in the oxygen consumption levels of the leatherback turtle, *Dermochelys coriacea*. Peaked patterns have been found for the Nile soft-shelled turtle, *Trionyx triunguis*, (Leshem, Ar, & Ackerman, 1991) and for the painted turtle *Chrysemy picta picta* (Peterson & Kruegl, 2005). The peaked pattern of oxygen consumption in *C. picta picta* was surprising, since it was the first documented case of a peaked oxygen consumption curve in the family Emydidae (Peterson & Kruegl, 2005).

The pattern of turtle development resembles that of precocial birds, with the oxygen consumption rate increasing exponentially for about 80% of development before slowing down towards the end of development (Gettinger, Paukstis, & Gutzke, 1984; Leshem, Ar, & Ackerman, 1991). The pattern of oxygen utilization can be explained by different stages of development in tortoises. Oxygen consumption increases exponentially after about the first third of the incubation period (Leshem et al, 1991; Thompson, 1991). The tortoise embryo is typically in the gastrula stage when it is laid (Ewert, 1979), making the oxygen needs of the embryo more limited during this period. Once the embryonic disk attaches to the egg, an opaque region, which eventually encompasses the entire egg, is formed (Chan, 1989). The opaque region is associated with the drying of the egg shell, which allows for quicker conduction of oxygen across the shell

(Thompson, 1989). The increase in the opaque region during development presumably allows the embryo to meet its rising metabolic demand by increasing the oxygen diffusion across the shell. The exponential stage of oxygen consumption correlates with the exponential increase in embryo mass during development (Ackerman, 1981b; Lynn & von Brand, 1945). The asymptote or decrease in the volume of oxygen in the egg is thought to indicate a halt in growth and the maintenance of the tortoise embryo (Leshem et al, 1991). Leshem et al (1991) found that the volume of oxygen consumed drops when 70% of the incubation period is completed, while the development of the embryo stabilizes after 80% of incubation. The discrepancy between the stabilization of the curves occurs because the smaller, growing embryo utilizes the same amount of oxygen as a full-sized embryo in a resting stage (Leshem et al, 1991).

While the physiological reason for the decrease in the volume of oxygen used by embryos is not disputed, the reason for the delayed hatching has two possible explanations. Thompson (1989) proposed the peaked pattern of oxygen consumption was a mechanism for promoting synchronized hatching in nests. Synchronized hatching is often seen in sea turtle nests, where developmental rates of the eggs varied with the difference in temperatures to which the top and bottom eggs were exposed (Thompson, 1989). Eggs nearer the surface of the nest were exposed to higher temperatures than the eggs at the bottom of the cavity for 75% of the day (Thompson, 1989). In species that did not have highly stratified temperatures in the nest or for whom synchronous hatching was not part of the survival strategy, the oxygen consumption of the eggs increased throughout incubation or asymptoted (Thompson, 1989). Thompson (1992) supported this idea by elucidating the sigmoidal pattern of oxygen consumption of the leatherback sea turtle, *Dermochelys coriacea*. The leatherback sea turtle had a regular thermal environment in its nests (Thompson, 1993). However, *Chrysemys picta picta* also exhibited a

peaked pattern (Peterson & Kruegl, 2005). The painted turtle overwinters in the nest, making a mechanism attributed to preventing premature exiting of the nest unnecessary (Peterson & Kruegl, 2005). Another possible explanation for the peaked or asymptoting pattern of development in turtles is the internalization of the yolk. Many turtles pip or hatch with a large amount of external yolk, which must be internalized (Ewert, 1991). The hatchlings will often stay in the nest after pipping, and the emergence from the egg is thought to assist in the internalization of the yolk (Ewert, 1979). In Testudinidae, the family containing gopher tortoises, the yolk sack is generally 30 to 50% the length of the plastron (Ewert, 1979), indicating that gopher tortoises should at the very least have a sigmoidal oxygen consumption pattern.

Many of the factors influencing the rate of oxygen consumption by embryos were controlled by the nest environment. The soil type of the nest controlled the amount of oxygen available (Prange & Ackerman, 1974), while factors such as cavity depth and the amount of vegetation cover controlled the temperature range to which the embryos were exposed (Wilson, 1998). Females of several turtle species have been shown to actively select their nesting site (Spencer & Thompson, 2003; Wilson, 1998). Nest sites selected by females produced higher embryo survivorship rates than did eggs placed in random sites (Wilson, 1998). No studies have conclusively linked nest-site selection to gopher tortoises, but females in Mississippi have been observed digging and subsequently abandoning nest cavities (personal observation). Tortoises in southwestern Georgia show a clear preference for open areas such as burrow aprons (Landers, Garner, and McRae, 1980). Because the soil in the western portion of *G. polyphemus*' range is characterized by higher clay content than the eastern range (Auffenberg and Franz, 1982; Epperson and Heise, 2003; Noel, 2006), the females' choices of soil have a much higher clay content. Noel (2006) asserts that high clay content, when paired with heavy rains, might lead to

suffocation of embryos. Limited gas exchange in experimental nests have been shown to yield high embryo mortality (Ackerman, 1981a).

In order for a causal link to be drawn between mortality in a nest and oxygen provided by different microhabitats, two pieces of information must be quantified. First of all, the levels of oxygen must be measured and shown to differ between the nests of the two soil types. Secondly, the oxygen needs of the embryos at different temperatures must be known. Because the nest temperature fluctuates in natural nests, a range of oxygen consumption measurements is necessary. This current study will answer the following questions: 1) what is the rate of oxygen consumption of gopher tortoise embryos at different temperatures throughout development and 2) do dead embryos consume a disproportionate amount of oxygen? Together with data on oxygen levels in nests in different soils (to be collected by other researchers), these data will allow us to determine whether and how frequently eggs in nests experience hypoxic or anoxic conditions.

### Chapter 3: Methods

The goal of this experiment is to quantify the oxygen requirements of developing gopher tortoise embryos at varying temperatures. In Mississippi, the gopher tortoise (*Gopherus polyphemus*) is classified as endangered and has been the focus of research to determine the cause of their decline. Noel (2006) found that while 60% of the eggs are intrinsically capable of hatching, only 17% hatch in the wild. The discrepancy indicates an environmental, or extrinsic, cause that is in part responsible for the low hatching success rate (Noel, 2006). This experiment will be conducted in conjunction with a graduate student from Dr. Carl Qualls' lab in order to elucidate possible limiting environmental factors. Information gathered on oxygen consumption

of the eggs from this study will later be coupled with the nest data from another concurrent experiment in the lab in order to determine if interstitial oxygen levels are adequate.

During the course of this experiment, three sites within Forrest and Perry counties of Mississippi will be searched for eggs. Eggs will be allocated between the two research projects, and the eggs participating in the oxygen consumption experiment will be taken to the University of Southern Mississippi (USM) for incubation. Once at USM, the oxygen consumption of each egg will be measured at regular intervals throughout development. The oxygen consumption of dead eggs will also be measured in order to see if decaying embryos in a nest may negatively impact interstitial oxygen levels.

### **Collection sites and egg selection**

The Desoto National Forest in Mississippi is home to the largest population of gopher tortoises in the western portion of their range (Noel, 2006). Three sites in the DeSoto National Forest will be used as a source for eggs. Two of the sites--training site 44 (T-44) in Camp Shelby and a site outside the town of McLaurin (McLaurin)--have the high clay soils that characterize most of the western range, while the Crossroads site has sandier soils. T-44 in Camp Shelby is a 900 ha gopher tortoise reserve characterized by low training activity and prescribed fires to maintain the environment (Noel, 2006). Within T-44, populations on the eastern-most road (31.0751°N, 89.1019°W) and on the western-most road (31.0730°N, 89.1315°W) will be searched for eggs (Noel, 2006). McLaurin (31.1492°N, 89.2023°W) is a 14.2 ha area of land that is burned every two to three years. Finally, eggs will also be collected from the Crossroads site (30.9535°N, 89.1101°W), which is a sandier site outside of Fruitland Park.

Each of the three sites will be examined for active and inactive burrows in the middle of

May. Burrows will be classified as active if the mouth has a half-arc shape, is clear of leaves and cobwebs, and has visible evidence of other tortoise activity, such as foot prints or feces (Wilson, Mushinsky, & Fischer, 1997). Burrows will be classified as inactive if they retain the half-arc shape but show no signs of recent occupancy, while burrows that are no longer arc-shaped will be designated as abandoned (Wilson, Mushinsky, & Fischer, 1997). Active and inactive burrows will be noted on a map and will be marked with flagging tape. Based on numbers from the 2006 field season, approximately 12 burrows are expected at T44E, 63 at T44W, 15 at McLaurin, and 32 at Crossroads.

Once every site has been thoroughly searched for burrows, the aprons of the active burrows will be hand-probed following guidelines described by Epperson and Heise (2003). Nest searches will be conducted daily from mid-May to the end of June. Inactive burrows will be checked at least once a week for evidence of new occupancy. When eggs are found in an apron, the nest will be carefully excavated, and all eggs will be removed. The position of the eggs will be noted to prevent any turning of the eggs. If an odd number of eggs are in the nest, one egg will be randomly assigned to the oxygen consumption experiment. The rest of the eggs will be split between artificial nest cavities in the DeSoto district and the Chickasawhay district of the Desoto National Forest for the interstitial gas experiment.

The eggs will be transported to USM within twenty-four hours of oviposition. The age of the egg will be estimated by the presence and size of a white spot at the top of the egg. The top of the egg will be marked with a grease pencil to ensure the original orientation is maintained and will be placed in a 0.95 L jar half filled with moistened vermiculite. The vermiculite will be prepared using 0.7g of water per gram of dry vermiculite to give a water potential of -200 kPa (Packard, Packard, Miller, & Boardman, 1987). The jar will be covered with plastic wrap held in

place by a rubber band (Noel, 2006) and placed in a padded cooler to minimize jostling and thermal stress during the drive back to USM. Because the average number of eggs per clutch is about five (Butler & Hull, 1996; Epperson & Heise, 2003; Noel, 2006) and a total of 18 clutches were found in the 2006 field season, an estimated 18 eggs will be available for use in this experiment.

### **Incubation and Oxygen Measurements**

Once at USM, each egg will be assigned to one of two environmental chambers (Model I-75A, Percival Scientific Inc.). The environmental chambers will have two different thermal regimes with varying temperatures in order to mimic natural nest conditions. Half the eggs will be incubated in a chamber with a thermal regime averaging to 28.3°C, while the other half will be incubated in a thermal regime averaging to 30.3°C. The first thermal regime should produce mostly males, and the second thermal regime should produce mostly females (Demuth, 2001). The temperatures in each chamber will be measured using Hobo-Temp data loggers (Onset Pro Series H08-031-08). Eggs will alternately be placed into each temperature group in order to make the selection process as random as possible.

Oxygen measurements will be taken in a third environmental chamber. Each egg will have its oxygen consumption measured at five different temperatures, which will be determined from nest temperature data. Using every egg for each temperature measurement eliminates the confounding factors of different genetics, egg size, and developmental rates. The measurements will be made at designated intervals measured from the date of oviposition. The oxygen consumption measurements will be conducted using either an open system (Ackerman, 1981b; Lynn & von Brand, 1945; Prange & Ackerman, 1974) or a closed system (Gettinger, Paukstis, & Gutzke, 1984; Leshem, Ar, & Ackerman, 1991; Peterson and Kruegl, 2005; Thompson 1992).

The closed system requires knowledge of the carbon dioxide and water composition of the air, as well as the volume of the container (Vleck, 1987). The draw-back to this system is that the volume of the egg must be subtracted from the volume of the container. Gopher tortoise eggs are not perfectly spherical, so error will be introduced in this measurement. In the open system, the difference between a base-line of ambient air and air that has been drawn through the chamber containing the organisms would be the amount of oxygen utilized by the organism in that period of time. In either case, the FoxBox system (Sable Systems) coupled with the RH-300 humidity sensor (Sable Systems) will be measuring the oxygen consumption. The FoxBox system measures both carbon dioxide and oxygen simultaneously, and also measure water vapor pressure, allowing correction of respiratory gas readings for the relative humidity in the gas samples.

Once the eggs hatch, the hatchlings will be kept in the lab until the yolk sac has been internalized and the hatchlings are mobile. The hatchlings will then be marked according to Cagle (1939). The hatchlings will then be released per the instructions of Tom Mann, Zoologist for the Mississippi Department of Wildlife, Fisheries, and Parks. In the 2006 field season, hatchlings from T44 were given to the Nature Conservancy Head Start project at Camp Shelby. Other hatchlings were released on the burrow apron from which they hatched.

Data collected by the FoxBox system will be recorded onto a separate computer for statistical analysis. Specific analytical techniques will be determined by whatever respirometry system (open or closed) is used. In order to be able to compare the oxygen consumption pattern of the gopher tortoise with other members of the Testudinia clade, the volume of oxygen consumption will be graphed against the days of incubation for each egg. The volume of oxygen consumed for each egg at each time interval will also be graphed in order to compare metabolic

rates at different temperatures. Finally, regression analysis will be used to generate mathematical models of the metabolic rate across temperatures and throughout development. The mathematical models will be used by related research to estimate the frequency of detrimental gas exchange in the nest. Detrimental gas exchange includes anoxic conditions and high levels of carbon dioxide.

### **Consent and Permitting**

Gopher tortoises are considered a threatened species by the federal government and an endangered species by the state of Mississippi (Wilson, Mushinsky, & Fischer, 1997). Permits to handle and conduct research on the gopher tortoises will be acquired from Mississippi Department of Wildlife, Fisheries, and Parks. Additional permission to conduct research on national forests lands will be obtained from Diane Tyrone, the district biologist of the DeSoto District. The requests for permits will be submitted in January to ensure their timely arrival. Permission to conduct the experiments on the eggs will also need to be obtained from the University of Southern Mississippi's Institutional Animal Care and Use Committee (IACUC).

### **Tentative Timeline**

In order to be able to complete this experiment on time, a protocol for using the gas analyzing equipment must be established well before May. My goal is to have the gas analyzer protocol established by February 2007. Starting in January 2007, the respirometry chamber will be designed. Preliminary gas analysis coupled with the respirometry chamber can be conducted in March and April in order to work out any problems with the system. The actual field research will be conducted from mid May to the end of June. From June until hatching, the oxygen measurements will be made. The nests in the field will be checked for any hatchlings from mid-August to the end of September, and any hatchlings from the oxygen measurements will be

released. Finally, the regression analysis of the data will begin in October, when the hatching season is finished.

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