ENVIRONMENTAL INFLUENCES ON THE CRITICAL OXYGEN TENSION, WATER BALANCE, METABOLISM, AND GROWTH OF REPTILIAN EMBRYOS

BY

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This work is dedicated to my parents, brothers, sisters,
my wife, Iris, and my son, Michael

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The effect of hypoxia on gas exchange of reptilian embryos was studied. Embryos of Alligator mississippiensis, Caretta caretta, Pseudemys nelsoni, Elaphe obsoleta quadrivittata, and Sceloporus undulatus undulatus maintained normal levels of oxygen consumption, $\dot{V}_{O_2}$, to some degree of hypoxia, indicating that they are metabolic regulators. A low $\dot{V}_{O_2}$ and a high eggshell conductance for O_2 explain how reptilian embryos maintain their $\dot{V}_{O_2}$ in hypoxic conditions. Hypoxic tolerance decreased during incubation, as indicated by an increase in the critical oxygen tension, P_c. The P_c increased presumably because oxygen transport capacity, reflected by the critical oxygen gradient, G_c, did not offset the increase of $\dot{V}_{O_2}$ during development.

Embryos of the Florida red-bellied turtle, P. nelsoni, were used as model organisms to further examine environmental influences on gas exchange and

physiological responses of embryos to hypoxia and flooding. Oxygen consumption and P_c of embryos were significantly affected by temperature. The Q_{10} values remained the same (Q_{10} = 2-3) at different incubation days, thus indicating that the thermal sensitivity of metabolism does not change during development. The P_c was temperature-dependent and increased in direct proportion to temperature at each incubation day. Oxygen consumption and P_c of embryos were not affected by egg hydration. Eggs incubated in 13 % gravimetric water content gained mass at a faster rate than those in 3 % gravimetric water content, and the respective egg masses were statistically different at day 30 and 39. However, oxygen consumption, critical oxygen tensions, and hatchling mass of the two groups were not different. Exposure to chronic hypoxia (10 % air) resulted in retarded growth, depressed metabolism, and reduced hatchling mass but comparable incubation period and hatchability. Embryos are flexible, structurally and physiologically, in enhancing oxygen transport to compensate for hypoxic effects. Simulated flooding has dramatic effects on water balance, embryonic metabolism and development of embryos. One-day submergence in water did not affect water balance and oxygen consumption of embryos, but did increase egg mortality. On the other hand, 3- and 6-day submergence treatments affected water balance, oxygen consumption and hatching success of eggs.

GENERAL INTRODUCTION

Eggs incubated underground are subjected to hypoxia (low oxygen tension) when O_2 concentration is reduced in the nest chamber or when embryos outgrow the O_2 diffusing capacity of their exchange systems (Ackerman 1977; Metcalfe et al. 1984). The O_2 concentration within a nest chamber depends on the balance between the rate of O_2 uptake of eggs and the rate of O_2 diffusion through soil (Ackerman 1977, 1981b; Packard and Packard 1989). Hypoxia that occurs in the crocodile Crocodylus, the green sea turtle Chelonia, and the loggerhead sea turtle Caretta nests during the second half of incubation is probably due to the large increase in oxygen consumption (Ackerman 1977; Lutz and Dunbar-Cooper 1984). In addition, microbial respiration could also deplete oxygen in the nest, as in incubating mounds of the brush turkey, Alectura lathami (Seymour et. al. 1986). Finally, wetting of the incubating media by rainfall (Chabreck 1975), flooding (Plummer 1976), and high tides, reduces soil O_2 diffusion (Kam 1988; Seymour et al. 1986) and might cause short-term, periodic hypoxia in nests.

Even if the nest chamber is normoxic (normal oxygen tension), embryos might experience internal hypoxia because of the limitation of the O_2 diffusing capacity of the eggshell. Partial pressure of oxygen (P_{O2}) in air space of chicken eggs decreases during incubation due to restricted diffusion across the eggshell (Temple and Metcalfe 1969; Tazawa 1980). Oxygen consumption ($\dot{V}_{O2}$) increases when eggs are exposed briefly to hyperoxia (Metcalfe et al.

1981; Stock et al. 1985; Stock and Metcalfe1987). It has been postulated that during late incubation, chick embryos outgrow the oxygen diffusing capacity of their gas exchange systems; thus, embryos become metabolic conformers during late incubation as found in megapode and chick eggs, in contrast to being metabolic regulators during early incubation (Visschedijk et al. 1980; Seymour 1985).

A metabolic regulator can maintain a constant $\dot{V}_{O_2}$ to some degree of hypoxia, whereas a metabolic conformer has $\dot{V}_{O_2}$ proportional to environmental P_{O_2} (Prosser 1973). It is not known whether reptilian embryos are metabolic regulators or conformers, or how their development influences metabolic regulation. Embryonic development is an energy-demanding process, and growing embryos consume more and more oxygen. Earlier studies on adult turtles (Belkin 1965), lizards (Boyer 1966), and plethodontid salamanders (Beckenbach 1975) demonstrated that increased metabolism due to elevated body temperature increases critical oxygen tensions (Pc, the P_{O_2} at which the oxygen consumption is first reduced) proportionally. This may not be true for embryos because their oxygen transport system, including chorioallantoic membrane and blood, develops as they grow and increases the ability to extract oxygen (Tazawa 1980). In theory, if the oxygen transport system did not develop as embryos grow, then the Pc of embryos would increase with increased metabolism. In contrast, if the oxygen transport system developed in concert with embryonic development, then developing embryos might tolerate the same hypoxic conditions even at a higher metabolic level, i.e, no resultant change in Pc.

Environmental variables such as temperature and hydric conditions also interact with embryonic development in determining the Pc of embryos. Adult reptiles increase their Pc in direct proportion to any given metabolic level as

body temperature changes (Belkin 1965; Boyer 1966). Direct and indirect evidence indicate temperature affects the metabolism of eggs (Zarrow and Pomerat 1937; Packard and Packard 1989; Deeming and Ferguson 1989), and the Pc presumably will increase in proportion to the $\dot{V}_{O_2}$. Also, reptilian eggs take up water from their surroundings under favorable conditions. The O_2 conductance of the egg is inversely correlated to the amount of water uptake, suggesting that O_2 transport capacity of embryos is reduced as they developed (Black et al. 1984).

If hypoxia does occur, what is its effect on embryonic metabolism and how do reptilian embryos respond? Chronic hypoxia causes slower growth, longer incubation time, and lower hatching success in embryonic turtles of Caretta caretta and Chelonia mydas (Ackerman 1981a). However, he did not measure the oxygen concentration in the artificial nests, and thus the hypoxic effects were not estimated. Further, physiological adjustments of embryos to minimize the hypoxic effect have not been studied.

One extreme case of hypoxia occurs when eggs are flooded. Reptilian eggs incubated underground are potentially subjected to flooding when water level is raised by rainfall, high tides, or other natural phenomena. Laboratory and field studies have demonstrated that flooding is a major cause of egg mortality of Crocodylus porosus (Webb et al. 1977), Alligator mississipiensis (Hines et al. 1968, Joanen 1969, Kushlan and Kushlan 1980), and Trionyx m. muticus (Plummer 1976). However, no attempt has been made to investigate flooding influence on water balance, metabolic rate, or embryonic development of eggs. When eggs are inundated, oxygen availability drops to a minimum level. Eggshells are saturated with water, which favors water exchange but not gas exchange. It is not known how embryos respond to a sudden change from an aerial environment to an aqueous environment; and for those embryos that

survive, it is unclear if flooding has any effect on embryonic development and metabolism.

The present study investigates gas exchange of reptilian embryos exposed to hypoxic conditions. Specifically, I compare the effect of acute hypoxia on $\dot{V}_{O_2}$ of embryos among five different species. Then I choose the Florida red-bellied turtle, Pseudemys nelsoni, as model organisms to investigate (1) the environmental influences on the critical oxygen tensions of eggs, (2) the physiological responses of embryos to hypoxia, and (3) the effect of flooding on water balance, embryonic metabolism and growth of embryos.

CHAPTER 1
COMPARATIVE STUDY ON THE CRITICAL OXYGEN TENSION OF REPTILIAN EMBRYOS

Introduction

Eggs incubated underground are subjected to hypoxia when O_2 concentration is reduced in the nest chamber or when embryos outgrow the O_2 diffusing capacity of their exchange systems (Ackerman 1977; Metcalfe et al. 1984). The O_2 concentration within a nest chamber depends upon the balance between the rate of O_2 uptake of eggs and the rate of O_2 diffusion through soil (Ackerman 1977, 1981b; Packard and Packard 1989). Hypoxia that occurs in Crocodylus, Chelonia, and Caretta nests during the second half of incubation is probably due to the large increase in oxygen consumption (Ackerman 1977; Lutz and Dunbar-Cooper 1984). In addition, microbial respiration could also deplete oxygen in the nest, as in incubating mounds of the brush turkey, Alectura lathami (Seymour et. al. 1986). Wetting of the incubating media by rainfall (Chabreck 1975), flooding (Plummer 1976), and high tides reduces soil O_2 diffusion (Seymour et al. 1986; Kam 1988) and might cause short-term, periodical hypoxia in nests.

Even if the nest chamber is normoxic, embryos might experience hypoxia because of the limitation of O_2 diffusing capacity of the eggshell. The P_{O_2} in air space of chicken eggs decreases during incubation due to restricted diffusion across the eggshell (Temple and Metcalfe 1969; Tazawa 1980). Also, embryonic oxygen consumption ($\dot{V}_{O_2}$) increases when the egg is exposed briefly to hyperoxia (Metcalfe et al. 1981; Stock et al. 1985; Stock and

Metcalfe1987). It has been postulated that during late incubation chick embryos outgrow the oxygen diffusing capacity of their gas exchange systems (Metcalfe et al. 1984); thus, embryos become metabolic conformers during late incubation, as found in megapode and chick eggs, in contrast to being metabolic regulators during early incubation (Visschedijk et al. 1980; Seymour 1985).

A metabolic regulator can maintain a constant $\dot{V}_{O_2}$ to some degree of hypoxia, whereas a metabolic conformer has $\dot{V}_{O_2}$ proportional to the environmental P_{O_2} (Prosser 1973). It is not known whether reptilian embryos are metabolic regulators or conformers, or how their development influences their metabolic regulation. Black et al. (1984) reported that P_{O_2} gradients across shells of python eggs decrease during incubation, suggesting that embryos are subject to hypoxia as are avian embryos (Tazawa 1980). Embryonic development is an energy-demanding process, and growing embryos consume more and more oxygen. Earlier studies on adult turtles (Belkin 1965), lizards (Boyer 1966), and plethodontid salamanders (Beckenbach 1975) showed that increased metabolism due to elevated body temperature increases critical oxygen tensions (P_c). However, this may not be true for embryos because their oxygen transport system, including chorioallantoic membrane and blood, develops as they grow and increases the ability to extract oxygen (Tazawa 1980). In theory, if the oxygen transport system did not develop as embryos grow, then the P_c of embryos would increase with increased metabolism. In contrast, if the oxygen transport system developed in concert with embryonic development, then developing embryos might tolerate the same hypoxic conditions even at a higher metabolic level, i.e., no resultant change in P_c.

The purpose of this chapter is to examine the metabolism of reptilian embryos as related to environmental oxygen and to investigate how embryonic development influences the metabolic regulation. Critical O_2 tension curves of five species of reptilian eggs were determined throughout incubation.

Materials and Methods

Animals

Sixteen to twenty-four eggs of each of five species, the American Alligator, Alligator mississippiensis, the loggerhead sea turtle, Caretta caretta, the Florida red-bellied turtle, Pseudemys nelsoni, the yellow rat snake, Elaphe obsoleta quadrivittata, and the southern fence lizard, Sceloporus undulatus undulatus, were used (Table 1-1). Caretta caretta eggs were collected directly after oviposition at Melbourne Beach, Florida. Elaphe o. quadrivittata and S. u. undulatus eggs were oviposited in laboratory terraria by gravid females collected in Alachua County, Florida. Alligator mississippiensis and P. nelsoni eggs were collected from local alligator nests, and one egg from each clutch was dissected and its age was estimated.

Eggs for each species were assigned equally to two or three boxes. All except A. mississipiensis eggs were half-buried in sand with 3% gravimetric water content (g water / g sand) and incubated at 30 °C. Alligator mississippiensis eggs, which were housed in the Fish and Wildlife Commission Laboratory at Gainesville, Florida, were half-buried in sphagnum moss with 90-130 % gravimetric water content and incubated at 32 °C. The boxes were covered with plastic sheets to reduce evaporative water loss, and water was added as necessary.

Oxygen consumption measurements at different P_{O_2}

The $\dot{V}_{O_2}$ was measured using a closed-system. Different sizes of glass jars (Kerr Glass Inc.) fitted with two rubber stoppers and three-way stopcocks were used as metabolic chambers. Chamber volume was determined by weighing the jar empty and filled with water, then dividing the difference between measurements by water density at room temperature.

Four to six eggs were randomly selected at early, middle, and late stages of incubation (Table 1-1). One day before each experiment, eggs were weighed to the nearest 0.001g, and the length and width were measured to the nearest 0.01 mm. An egg was placed in a cup inside a metabolic chamber containing 5 ml of distilled water and a piece of wet filter paper to keep the chamber at 100% relative humidity. The chambers were equilibrated at 30 °C overnight.

At the onset of the experiment, several metabolic chambers were submerged in a water bath at 30 °C except for the stopcocks . One empty chamber served as a control. The chambers were connected via stopcocks by Tygon tubing. Gas mixtures were made from nitrogen and air by means of a Digamix gas mixing pump (Type M/300 a) and humidified by bubbling through a gas-sealed flask containing distilled water at 30 °C. Embryos were allowed to equilibrate at each gas mixture for 15-20 min after the chambers were flushed. Preliminary experiments demonstrated that 15-20 min of equilibration time produced the same results as 40 and 60 min of equilibration time. Metabolic chambers were then re-flushed thoroughly, and the three-way stopcocks of each chamber were closed quickly. The interval for gas sampling depended upon the developmental stage of embryos such that no more than 1-2% of the total oxygen was consumed (Vleck 1987).

About 15-20 min before gas sampling an Applied Electro-chemistry S-3A O_2 analyzer was flushed with the gas mixture until a baseline was obtained on a chart recorder and was maintained during measurements. Because the delivery rate from the mixing pump was higher than needed, the gas mixture was sampled by a pump located downstream with a smaller Tygon tubing inside the delivery tubing. The gas mixture was directed through soda lime and silica gel to absorb CO_2 and water vapor respectively before being fed into the O_2 analyzer.

A gas sample from each chamber was taken using two 60 ml gas tight syringes, each tipped with a three-way stopcock. One of these syringes was filled with 25 ml of saturated gas mixture. The syringes were fitted on the stopcocks of the chamber and clamped tightly onto an iron stand. As the 25 ml gas mixture in one syringe was injected gradually into the chamber, 25 ml of gas mixture was withdrawn simultaneously from the chamber into the other syringe. This simultaneous 'push and pull' mixing movement was repeated about ten times before a final gas sample was withdrawn and injected quickly into the oxygen analyzer to measure oxygen concentration. This sampling technique allowed the withdrawal of gas samples from the metabolic chamber without creating negative pressures.

Immediately after the O_2 concentration of all chambers was measured, eggs were subjected to the next gas mixture, ranging from 90 to 20% air, and the oxygen concentrations were determined by repeating the above protocols. Each new gas mixture was 10 or 20% lower in air content than the previous treatment. Every experiment was started at the same time of a day and completed in the same day. A complete experiment usually lasted 10-16 h. Each treatment did not last more than 2-3 h, and each egg was treated with at least six gas mixtures at each of the three stages of incubation.

The egg volume of ellipsoid eggs was calculated by use of the equation, $V=4/3\,\pi\, a^2b$, where V is volume (ml), a is the shortest diameter (cm), and b is the longest diameter (cm); whereas volume of spherical eggs was calculated by the equation, $4/3\,\pi\, r^3$, where r is the radius (cm). Oxygen consumption, $\dot{V}_{O_2}$ (ml STPD/ h·egg), was calculated using the equation, $\dot{V}_{O_2} = V(FI_{O_2}-FE_{O_2})/(1-FE_{O_2})t$, where V is the dry, CO_2-free air (ml), FI_{O_2} and FE_{O_2} are the initial and final oxygen fraction of a measurement (%), and t is the duration (h) from start to end of a measurement (Vleck 1987).

Experimental analysis

The surface areas of spherical eggs and ellipsoid eggs were respectively determined using the equation $A=4\,\pi\, r^2$, where A is surface area (cm^2) and r is radius (cm) and the equation $A=2\,\pi\, b^2 + 2\,\pi\, (ab/e)(\sin^{-1}e)$, where a = 1/2 length of the egg, b = 1/2 width of the egg, and eccentricity $e = [1-(b/a)^2]^{0.5}$ (Ackerman et al. 1985) respectively.

Measurements from eggs that did not hatch were discarded from analysis. Three C. caretta eggs were repeatedly used on days 39 and 45. The results were not different from non-repeated measurements; therefore, data were pooled. The $\dot{V}_{O_2}$ of two S. u. undulatus eggs were measured at day 18 in one chamber, and the average value was used. Experimental eggs hatched at the same time as controls and no external deformities were visible.

The critical oxygen tension, P_c (the P_{O_2} at which the oxygen consumption is first reduced) and the critical oxygen gradient, G_c (the slope of regression line below the P_c) were determined using a BASIC program that fits a two-segmented straight line (Yeager and Ultsch 1989). These data were analyzed individually and averaged. All means are expressed as mean (± SE) unless otherwise noted. Reduced major axis regression analyses were

used because variables in regression analyses were random measurements of a population with error.

Results

All embryos maintained $\dot{V}_{O_2}$ to some degree of hypoxia, although the degree of hypoxic tolerance varied among species (Figs. 1-1 to 1-5; Table 1-2). Sceloporus. u. undulatus was the most hypoxic-tolerant species, maintaining its $\dot{V}_{O_2}$ when P_{O_2} was 64 mmHg at day 25 of incubation. In contrast, C. caretta had the lowest hypoxic tolerance and did not maintain its $\dot{V}_{O_2}$ when P_{O_2} was lower than 123 mmHg following day 22 of incubation.

The degree of hypoxic tolerance of embryos decreased significantly during incubation except in C. caretta, as indicated by the increase of the critical oxygen tensions (P_c) (Table 1-2). The $\dot{V}_{O_2}$ and G_c also increased significantly during incubation, but the latter increased at a slower rate than the former. In some cases, the rate of increase of G_c slowed down at late incubation. For example, the $\dot{V}_{O_2}$ of P. nelsoni increased 249%, 191%, and 71% from day 12 to 26, day 26 to 34, and day 34 to 40 respectively, but the G_c increased 222%, 132%, and 37 % at the same periods (Table 1-2).

The correlation between log surface area/volume ratio (S/V) of eggs and log P_c was time-dependent (Table 1-3). The P_c was significantly correlated with S/V at early ($P < 0.01$) but not at middle and late the incubation period ($P > 0.05$). Further, the relative $\dot{V}_{O_2}$ ($\dot{V}_{O_2}$ at any incubation period expressed as a fraction of that at late the incubation period) was calculated to compare interspecifically the sensitivity of P_c to metabolic rate (Table 1-4). The P_c of S. u. undulatus was most sensitive to $\dot{V}_{O_2}$, whereas A. mississippiensis, E. o.

quadrivittata, and P. nelsoni were the least sensitive. At any given level of relative V_{O_2}, C. caretta had a P_c higher than that of the other species.

Discussion

Effects of development on oxygen consumption and P_c

The fact that reptilian embryos could maintain constant $\dot{V}_{O_2}$ over a range of environmental P_{O_2} during development indicates that they are metabolic regulators (Figs. 1-1 to 1-5). Different patterns have been reported for bird embryos in that embryos of the Mallee fowl, Leipoa ocellata, are metabolic regulators during early and middle incubation, but become metabolic conformers at late incubation (Seymour 1985). Embryos of chickens at day 16-19 could not maintain normal rates of metabolism when exposed to acute hypoxia (Visschedijk et al. 1980; Ar et al. 1991). A similar finding has been reported for the Canada goose (Branta canadensis) where embryos could not maintain normal metabolism when air cell oxygen tensions were reduced more than 5 mmHg (Snyder et al. 1982). But results from that same study also show that embryos of the bar-headed goose (Anser indicus) are metabolic regulators and have critical oxygen tensions 33 mmHg lower than that of the Canada goose. It appears that eggs of the bar-headed goose, laid at high altitude (as high as 5500 m), possess genetic adaptations which allow them to maintain normal oxygen consumption in hypoxic conditions (Snyder et al. 1982).

Reptilian embryos tolerate hypoxia better than bird embryos, and this difference is mainly attributed to the low $\dot{V}_{O_2}$ of embryos (Dmi'el 1970; Ackerman 1980;1981b) and, in some cases, the high O_2 conductance of

reptilian eggs (Ackerman and Prange 1972; Lutz et al. 1980; Feder et al. 1982). Bird and reptilian eggs with similar masses have different $\dot{V}_{O_2}$; for example, the $\dot{V}_{O_2}$ of the green turtle (Chelonia mydas) is only one-fourth that of chicken eggs at late incubation (Ackerman 1980). This difference is due to a lower incubation temperature and rate of growth in green turtle eggs (Ackerman 1980). Conversely, O_2 conductance of reptilian eggs varies among shell types and egg hydrations (Lutz et al. 1980; Feder et al. 1982; Black et al. 1984). American crocodile (Crocodylus acutus) eggs have a rigid eggshell which contributes the majority of the resistance to oxygen diffusion. Oxygen permeability of moist and dry eggshell-membrane complexes of C. acutus is similar to that of chicken eggs (Lutz et al. 1980). In contrast, the moist and dry eggshell-membrane complexes of flexible eggs, as found in the snapping turtle (Chelydra serpentina), have O_2 permeability ten times higher than that of chicken eggs (Feder et al. 1982).

The increase of P_c during incubation in this study resembles a finding previously reported for Mallee fowl eggs. This similarity suggests that both Mallee fowl and reptilian eggs have difficulty taking up sufficient oxygen to maintain their metabolism. Any P_c level can be described by the interaction of $\dot{V}_{O_2}$ and G_c. Embryonic development increases $\dot{V}_{O_2}$ which increases the P_c, whereas circulatory and respiratory developments promote the G_c which reduces the P_c. If oxygen diffuses across the respiratory surface at the maximum rate at a P_{O_2} below the P_c, G_c is a measure of the maximum overall oxygen transport capability (Belkin 1965; Beckenbach 1975; Bradford and Seymour 1988). An increase of P_c over incubation indicates that G_c does not develop in concert with embryonic development; in other words, although the G_c increases during development, it approaches its upper limit at late

incubation such that it cannot catch up with the pace of the increase of $\dot{V}_{O_2}$. Therefore, the embryos have to increase their P_c at a higher metabolic level.

Evidence suggests that the inability of G_c to match the increased $\dot{V}_{O_2}$ is due to diffusion limitation of the gas exchange surface. The P_c of hatchlings (P. nelsoni, 70±4.23 mmHg, n=4; C. caretta, 44.5±1.80 mmHg, n=5) is significantly lower than those of embryos at late incubation (Table 1-2) ($P<0.001$). The principal factor responsible for these differences may be the types of gas exchange organs (lung vs chorioallantoic membrane) (personal observation). A similar finding has been reported for the frog, Pseudophryne bibroni, in that eggs have much higher critical oxygen tensions than tadpoles (111 and 31 mmHg respectively, Bradford and Seymour 1988). This difference is due to the fact that tadpoles can ventilate gills and surface area to increase oxygen uptake in response to hypoxia, whereas eggs cannot. An earlier study of Burmese python eggs showed that the P_{O_2} beneath the eggshell decreases during development, suggesting that the eggshell-membrane complex poses a significant resistance to O_2 diffusion (Black et al. 1984). Similar results were found in chicken eggs where the air cell P_{O_2} drops during incubation due to restricted diffusion through the shell (Tazawa 1980). The oxygen diffusing capacity of eggs reaches a plateau at late incubation, and the supply of oxygen to tissues is maximized (Temple and Metcalfe 1970; Metcalfe et al. 1979; Metcalfe et al. 1984; Ar et al. 1991). Thus, when embryos are exposed to hypoxia, they are no longer able to maintain normal rates of oxygen consumption and become metabolic conformers (Visschedijk et al. 1980; Ar et al. 1991).

Interspecific comparisons of P_c

The P_c of turtle eggs did not differ from that of squamate and alligator eggs, which is opposite to the observations on adults. The P_c of adult turtles is about 8-24 mmHg when compared to that of lizards, snakes, and crocodilians of about 70 mmHg (Bennett 1976; Ultsch and Anderson 1988). Adult turtles exhibit a larger change in ventilation than do other reptiles when exposed to hypoxia (Boyer 1966; Glass et al. 1983), and this types of adjustment is not possible in eggs where oxygen is exchanged via diffusion over the chorioallantoic membrane.

The P_c of eggs was significantly correlated with S/V ratio at early incubation in that smaller eggs had a lower P_c than did larger eggs (Table 1-3). At a given relative $\dot{V}_{O_2}$ level, smaller eggs had larger G_c than larger eggs; thus more oxygen diffused across the eggshell in a given amount of time. Similar findings have been reported for lungless salamanders where the P_c is correlated with S/V ratio so that a smaller one has a lower P_c than a larger one (Beckenbach 1975). Interestingly, the correlationship between S/V ratio and P_c of eggs decreased over the incubation period and became insignificant during middle and late incubation (Table 1-3). This difference may relate to possible differences in development of respiratory and circulatory systems, water relation of eggs and other factors.

In this study, the sensitivity of P_c to metabolic rate differs among species (Fig. 1-6). The P_c of S. u. undulatus increased 83% from day 25 to day 42, whereas those of C. caretta and A. mississippiensis increased 9% from day 22 to day 45 and 32% from day 29 to day 56 respectively. The P_c of S. u. undulatus may be more sensitive to $\dot{V}_{O_2}$ than those of other species is because it has a higher water uptake. Eggs of S. u. undulatus increased in mass and attained 150.2 $\pm$ 5.78 % of initial mass at day 42, which is about 5-50 fold higher

when compared to eggs of other species (E. o. quadrivittata, 33.7 ± 1.18 % at day 47, C. caretta, 5.9 ± 0.91 % at day 45, A. mississippiensi, 2.84 ± 1.22 % at day 57, and P. nelsoni,12.99 ± 2.00 % at day 40). The tremendous amount of absorbed water in S. u. undulatus presumably forms a diffusion barrier which significantly reduces the O_2 conductance (Black et al. 1984) and increases P_c. The large change of P_c, subsequently, reduces the correlation between the P_c and S/V ratio (Table 1-3).

Ecological Considerations

All five species exhibited a similar pattern of metabolic regulation during incubation, i.e., increasing dependence of embryonic oxygen consumption on ambient P_{O_2}. The similarity of P_c of embryos between species late in incubation suggests hypoxic tolerance of reptilian embryos is independent of shell types and egg size. Also, the ability of embryos to tolerate hypoxia does not correlate with their habitats. For example, C. caretta embryos are the least hypoxia tolerant species even though they clearly experience hypoxic stress when the P_{O_2} of nest chambers decreases rapidly during the second half of incubation (Ackerman 1977)(Table 1-2). In contrast, E. o. quadrivittata and S. u. undulatus embryos are the most hypoxia tolerant species even though they probably do not experience hypoxic stress during incubation. These species are shallow nesters, and they lay eggs in hollow, rotting logs and vegetation, under railroad ties, beneath rocks, at the base of grass clumps and sawdust piles (Ashton and Ashton 1985, 1988; Ernest and Barbour 1989).

However, the generalizations about the ecological adaptation of reptilian embryos to hypoxic conditions are far from resolved due to extremely limited information concerning the responses of embryos to chronic hypoxia. Earlier studies on embryos of other animals show much flexibilities in adapting to their

hypoxic environments structurally or physiologically. Bullfrog tadpoles exhibit morphological changes in response to chronic hypoxia, including thinning the blood-water barrier by 50% and vasoproliferation in the skin, both of which probably facilitate gaseous diffusion (Burggren and Mwalukoma 1983). In chicken eggs, chronic hypoxia induces vasoproliferation in the chorioallantoic membrane, and thus increases surface area for gas exchange (Dusseau and Hutchins 1988; Strick et al. 1991). Exposure to hypoxia also increases hemoglobin-oxygen affinity in chicken and domestic fowl embryos (Black and Snyder 1980; Baumann et al. 1983) and heart mass of domestic chicken and Canada goose hatchlings (Black and Snyder 1980; Snyder et al. 1982). Hypoxia probably induces a redistribution of blood flow to embryonic tissue such that blood is preferentially distributed to an area with a high priority (e.g. heart) than to an area with a low priority (e.g. limb muscle). As a result, chicks that hatched in the hypoxic conditions have heavier hearts and lighter gastrocnemius muscles when compared to those hatched in the normoxic conditions, even though their body masses are the same (Black and Snyder 1980).

Table 1-1 Initial mass, incubation time, sample size, and sampling day of five species. Eggs were incubated at 30 °C with the exception of A. mississippiensis eggs (32 °C.). Egg mass of A. mississippiensis and P. nelsoni was measured at day 29 and 12 respectively. Values are reported as mean with standard error in parenthesis.

Species	no. egg	Initial egg mass (g)	Inc. time (days)	Sampling day (sample size)
A. mississippiensis	18	84.25 (1.39)	60 (0.3)	29(4), 40(4), 56(5)
C. caretta	24	43.68 (1.50)	57 (0.7)	22(5), 32(5), 39(6), 45(5)
P. nelsoni	24	13.02 (0.19)	50 (0.4)	12(4), 26(6), 34(4), 40(4)
E. o. quadrivittata	16	7.33 (0.09)	61 (0.7)	14(4), 31(4), 47(4)
S. u. undulatus	20	0.336 (0.004)	44 (0.3)	16(6), 33(6), 42(5)

Table 1-2. Critical oxygen tensions (P_c), critical oxygen gradients (G_c), and oxygen consumptions ($\dot{V}_{O_2}$) of five species. ANOVA tests show that all P_c, $\dot{V}_{O_2}$, and G_c of each species are at least significantly different at the 0.05 level over incubation with the exception of P_c of C. caretta ($P > 0.05$). Values are reported as mean with SE in parenthesis.

SPECIES	Age (Days)	P_c (mmHg)	$\dot{V}_{O_2}$ (ml/h)	$G_c \times 10^{-3}$ (ml/h mmHg)
A. mississippiensis	29	86.6 (3.80)	1.04 (0.11)	12.11 (2.11)
	40	103.8 (1.18)	2.91 (0.27)	25.59 (2.46)
	56	114.8 (5.28)	9.43 (0.58)	80.6 (2.28)
C. caretta	22	123.6 (3.23)	0.33 (0.01)	2.81 (0.10)
	32	122.6 (4.16)	1.32 (0.03)	12.35 (0.49)
	39	127.5 (4.65)	2.99 (0.10)	29.50 (1.72)
	45	135.8 (4.96)	4.60 (0.27)	42.85 (3.49)

Table 1-2--continued

SPECIES	Age (Days)	P_c (mmHg)	$\dot{V}_{O_2}$ (ml/h)	$G_c \times 10^{-3}$ (ml/h mmHg)
P. nelsoni	12	74.6 (7.56)	0.11 (0.001)	1.48 (0.08)
	26	86.0 (4.23)	0.39 (0.01)	4.76 (0.39)
	34	106.3 (2.41)	1.13 (0.02)	10.96 (0.36)
	40	120.5 (0.94)	1.93 (0.04)	14.98 (0.03)
E. o. quadrivittata	14	100.2 (3.12)	0.37 (0.003)	2.57 (0.19)
	31	108.0 (2.75)	0.63 (0.01)	5.24 (0.19)
	47	117.6 (4.09)	1.16 (0.05)	9.20 (0.50)
S. u. undulatus	16	64.3 (7.30)	0.05 (0.003)	0.52 (0.11)
	33	92.1 (2.51)	0.09 (0.002)	0.76) (0.01)
	42	117.0 (4.52)	0.14 (0.002)	1.14 (0.08)

Table 1-3. Reduced major axis regressions for the critical oxygen tension (P_c) in relation to the surface area/volume ratio of eggs. Data for all species were pooled at each incubation stage. Data of day 39 of C. caretta and day 34 of P. nelsoni were omitted. N = sample size; r = correlation coefficient ; P = probability of the correlation coefficient.

	N	Regression equations	r	P
Early incubation	18	Log P_c = 1.79 - 0.523 Log S/V	0.64	<.01
Middle incubation	24	Log P_c = 1.86 - 0.395 Log S/V	0.32	>.05
Late incubation	23	Log P_c = 2.02 - 0.195 Log S/V	0.20	>.05

Table 1-4. Reduced major axis regressions for the critical oxygen tension (P_c) in relation to the relative oxygen consumption ($R\dot{V}_{O_2}$)of eggs. N = sample size; r = correlation coefficient; P = probability of the correlation coefficient.

	N	Regression equations	r	P
A. mississippiensis	12	$\text{Log } P_c = 2.07 + 0.145 \text{ Log } R\dot{V}_{O_2}$	0.85	<.001
C. caretta	21	$\text{Log } P_c = 2.14 + 0.075 \text{ Log } R\dot{V}_{O_2}$	0.35	>.05
P. nelsoni	18	$\text{Log } P_c = 2.08 + 0.208 \text{ Log } R\dot{V}_{O_2}$	0.84	<.001
E. o. quadrivittata	11	$\text{Log } P_c = 2.08 + 0.178 \text{ Log } R\dot{V}_{O_2}$	0.78	<.01
S. u. undulatus	12	$\text{Log } P_c = 2.09 + 0.770 \text{ Log } R\dot{V}_{O_2}$	0.90	<.001

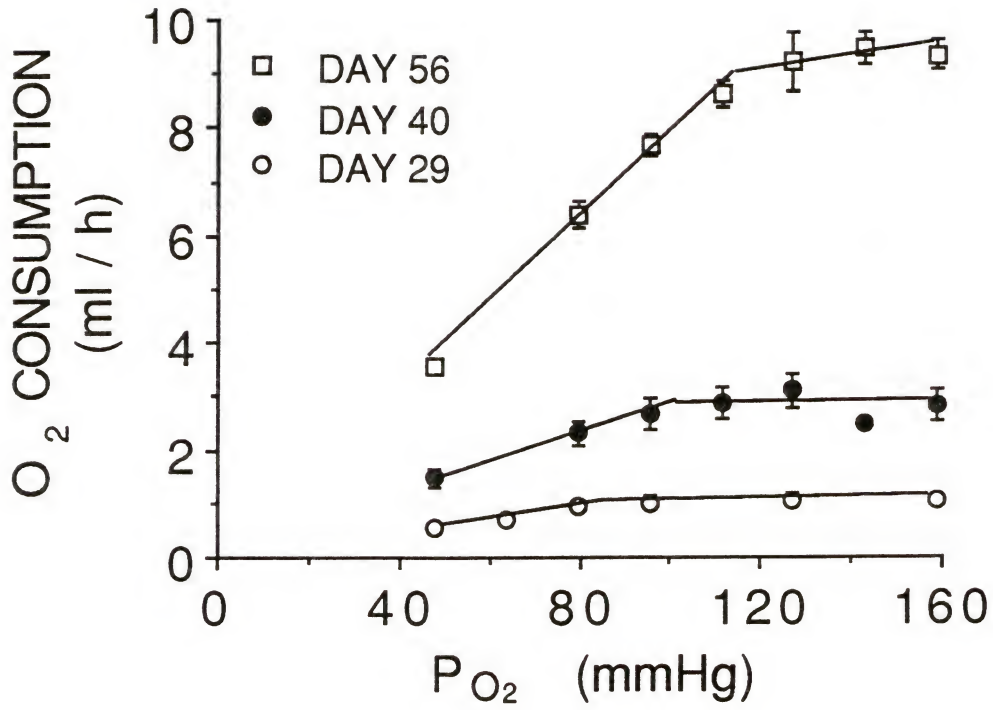

Figure 1-1. Oxygen consumption of Alligator mississippiensis embryos as a function of oxygen tension at different incubation days. Values are means ± SE.

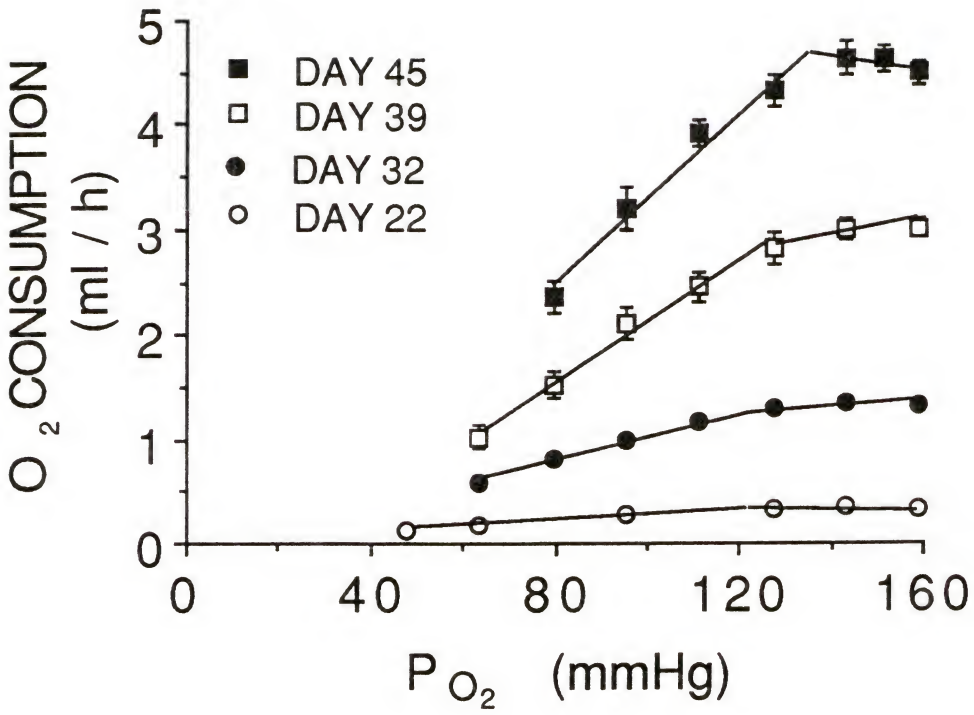

Figure 1-2. Oxygen consumption of Caretta caretta embryos as a function of oxygen tension at different incubation days. Values are means ± SE.

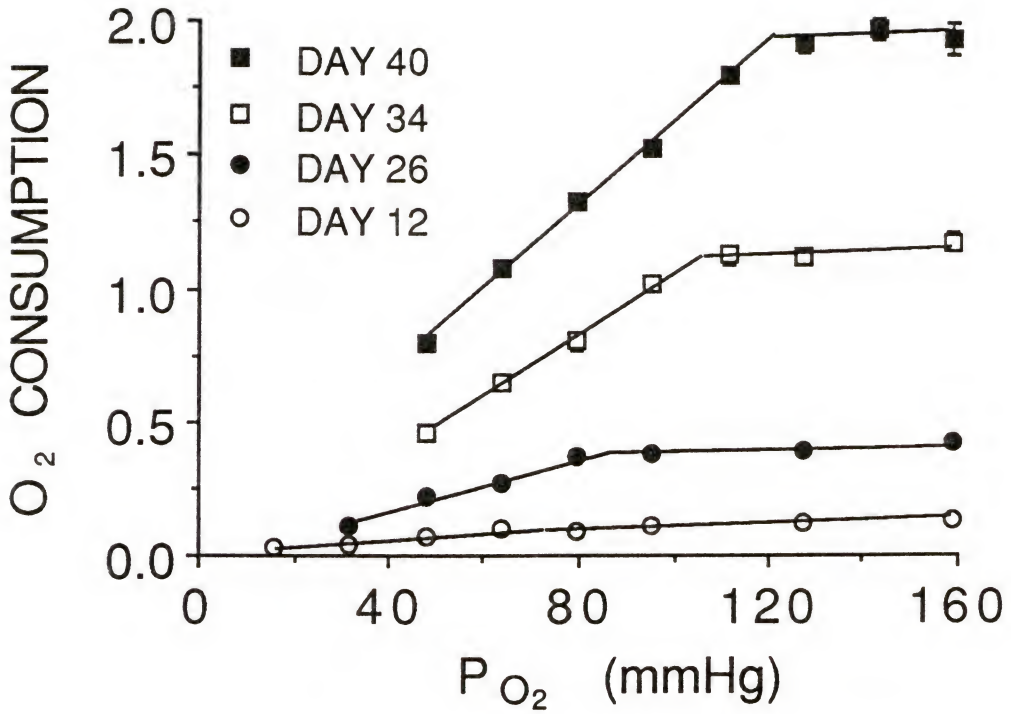

Figure 1-3. Oxygen consumption (ml / h) of Pseudemys nelsoni embryos as a function of oxygen tension at different incubation days. Values are means ± SE.

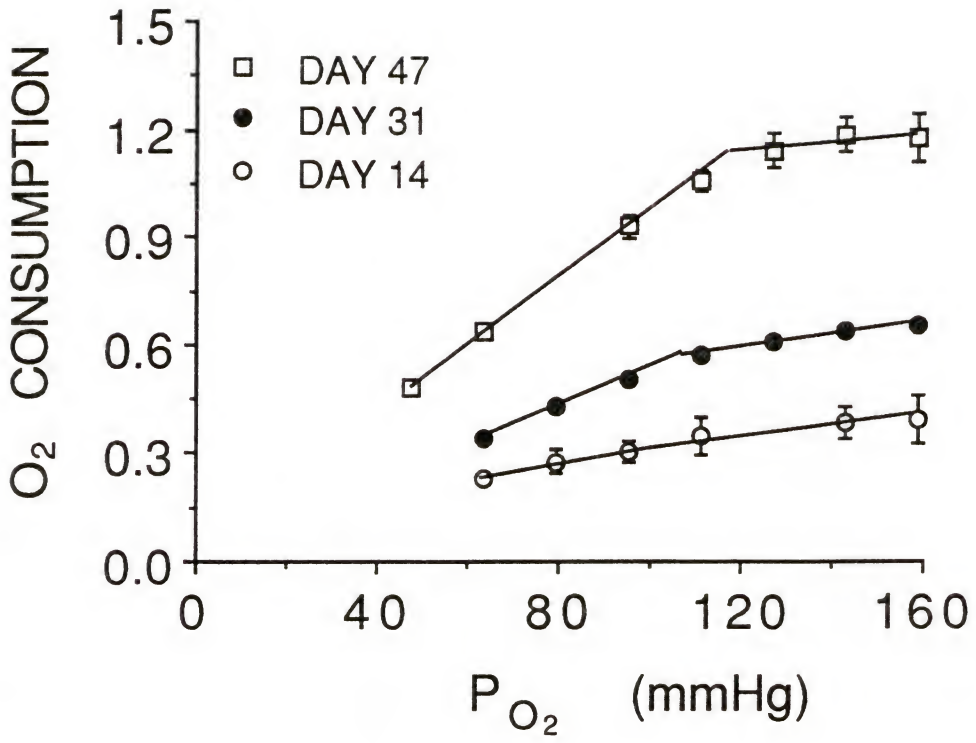

Figure 1-4. Oxygen consumption (ml / h) of Elaphe obsoleta quadrivittata embryos as a function of oxygen tension at different incubation days. Values are means ± SE.

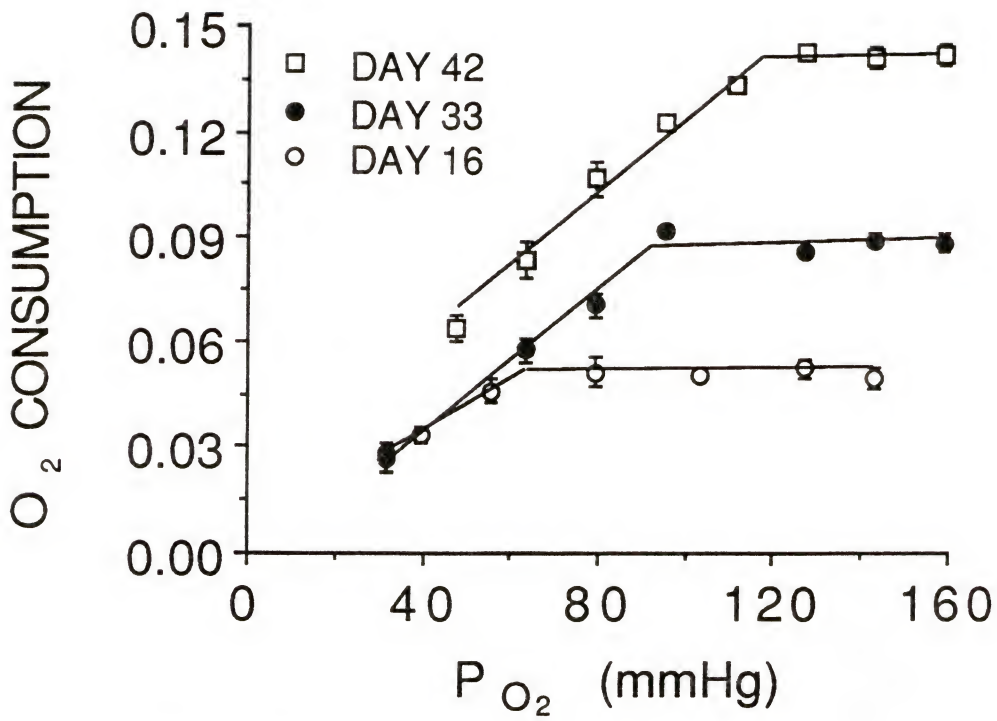

Figure 1-5. Oxygen consumption (ml / h) of Sceloporus undulatus undulatus embryos as a function of oxygen tension at different incubation days. Values are means ± SE.

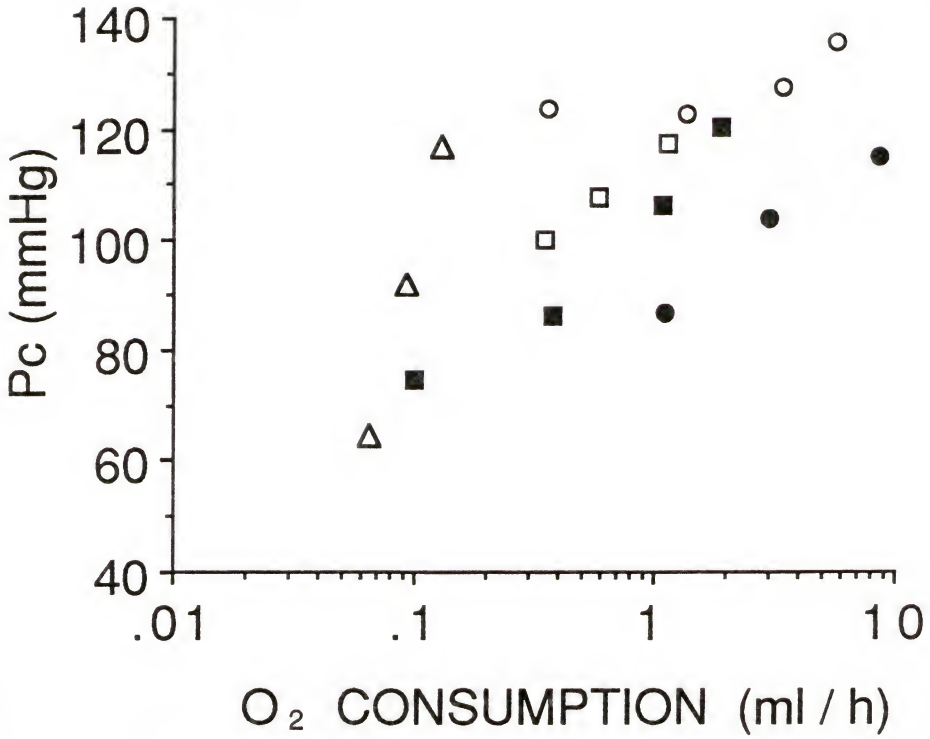

Figure 1-6. Relationship between critical oxygen tension (P_c) and oxygen consumption in embryos of reptilian eggs. A. mississippiensis (closed circles), C. caretta (open circles), P. nelsoni (closed squares), E. o. quadrivittata (open squares), and S. u. undulatus (open triangles).

CHAPTER 2
TEMPERATURE EFFECTS ON METABOLISM AND CRITICAL OXYGEN TENSION OF FLORIDA RED-BELLIED TURTLE (PSEUDEMYS NELSONI) EMBRYOS

Introduction

Many female red-bellied turtles (Pseudemys nelsoni) that are found in wetlands or wet prairies in Florida lay their eggs in nest mounds of the American alligator (Alligator mississippiensis). Most alligator nest mounds are dome-shaped and in close proximity to permanent water (Goodwin and Marion 1978). Oxygen content in a nest cavity depends on nest location and rainfall during the nesting season and could be reduced by microbial respiration, egg metabolism, and a low gas conductance of the nest due to wetting of incubation medium by rainfall or flooding.

The hypoxic tolerance of red-bellied turtle embryos has been assessed by measuring the critical oxygen tension (see Chapter 1). Turtle embryos are metabolic regulators during incubation, i.e., they can maintain a constant level of aerobic metabolism over a range of P_{O2}. Hypoxic tolerance, however, decreases during incubation, as indicated by an increase in critical oxygen tension, P_c, suggesting that the hypoxic tolerance is influenced by embryonic development.

The hypoxic problem is complicated by the thermal condition of nest mounds. About 54% of nests are located in the open field, and receives direct solar radiation during the day (Goodwin and Marion 1978). Depending on the location in the nest mound, the turtle eggs could experience a wide range of

temperatures. The influence of temperature on hypoxic tolerance of reptilian embryos has not been studied. Lungless plethodontids decrease their hypoxic tolerance in direct proportion to any given metabolic level as body temperature changes (Beckenbach 1975). In contrast, the hypoxic tolerance of some freshwater fishes is independent of temperature, suggesting the presence of adaptive adjustment(s) in response to temperature changes (Ultsch et al. 1978; Ott et al. 1980).

The objectives of this study were to investigate the effects of temperature on the hypoxic tolerance and metabolism of embryos of the Florida red-bellied turtle. Hypoxic tolerance was determined by measuring the critical oxygen tension of embryos. Embryonic metabolism was also reported at different temperatures to reveal the thermal sensitivity of metabolism of embryos during development.

Materials and Methods

Animals

A total of three clutches of red-bellied turtle eggs were collected from alligator nests in Florida during June and July, 1991. One egg from each clutch was opened to estimate age. Twenty eggs were assigned to each of three temperatures: 22, 27 and 32 °C. All eggs were half-buried in sand with 4% gravimetric water content and incubated at 30 °C. Boxes were covered with plastic sheets to reduce evaporative water loss, and water was added as necessary.

Oxygen consumption measurements at different P_{O_2}

The critical oxygen tension was determined by a method described elsewhere (see chapter 1). Briefly, each egg was placed in a cup inside a metabolic chamber containing 5 ml of distilled water, and the P_c was determined at the assigned temperature. Two metabolic chambers were submerged in each of the three water baths at 22, 27, or 32 °C for 3 hours before measurements were taken. Chambers were flushed and reflushed with a humidified gas mixture before stopcocks were closed. A 30 ml gas sample was collected from each chamber at the end of each measurement, and the final oxygen fraction was determined by injecting the sample into an Applied Electro-chemistry S-3A O_2 analyzer. Immediately after the O_2 concentration of all chambers was measured, eggs were subjected to the next gas mixture, ranging from 90 to 10% air, and the oxygen concentrations were determined by repeating the above protocol. Each new gas mixture was 5 to 20 % lower in air content than the previous treatment. Oxygen consumption, $\dot{V}_{O_2}$ (ml. STDP / h egg), was calculated using the equation, $\dot{V}_{O_2} = V(FI_{O_2}-FE_{O_2})/(1-FE_{O_2})t$, where V is the dry, CO_2-free air (ml), FI_{O_2} and FE_{O_2} are the initial and final oxygen fraction of a measurement, and t is the duration (h) from start to end of a measurement (Vleck 1987).

Experimental analysis

The critical oxygen tension, P_c (the P_{O_2} at which the oxygen consumption is first reduced) was determined using a BASIC program that fits a two-segmented straight line (Yeager and Ultsch 1989). Data were analyzed individually and averaged. Oxygen consumption was adjusted using analysis of covariance (ANCOVA) and the initial mass was used as covariate. Data

were analyzed individually and then averaged. All means are expressed as mean (± SE) unless otherwise noted.

Results

The critical oxygen tension curves of red-bellied turtle embryos at different temperatures and three incubation days are illustrated in Figures 2-1 to 2-3. Embryos at all treatments maintained $\dot{V}_{O_2}$ to some degree of hypoxia, although the hypoxic tolerance varied among temperature treatments and developmental conditions. Embryos exposed to 22 °C at day 39 were the most hypoxia-tolerant individuals, maintaining constant metabolism when P_{O_2} was at or above 47.7 mmHg. In contrast, embryos that were exposed to 32 °C at day 39 had the lowest hypoxic tolerance, and metabolism declined when P_{O_2} was lower than 117 mmHg.

A two-way ANOVA shows that oxygen consumption was significantly different among incubation days and temperatures ($P < 0.001$ and $P < 0.001$ respectively) (Fig. 2-4 and 2-6). Embryos increased $\dot{V}_{O_2}$ linearly during development at all three temperatures. Embryos exposed to high temperatures increased $\dot{V}_{O_2}$ faster than when exposed to low temperatures, suggesting an interaction between the two variables ($P < 0.01$)(Fig. 2-6). At a given incubation day, increasing temperature also produced a linear increase in $\dot{V}_{O_2}$ (Fig. 2-5).

Similar analysis shows that P_c was significantly different among temperatures ($P < 0.001$, Fig. 2-5) but similar among incubation days ($P = 0.804$, Fig. 2-7). Embryos exposed to 27 and 32 °C showed a slight increase or no change, respectively, in the P_c during development. In contrast, the P_c

of embryos exposed to 22 °C decreased during development (Fig. 2-7). Increasing temperature increased the P_c at each day of incubation (Fig. 2-5).

The sensitivity of P_c to $\dot{V}_{O_2}$ was highest when embryos were 16 days old, followed by embryos of 30 and 39 days (Fig. 2-8). The Q_{10} values, that were between 2-3, were higher at low temperatures than at high temperatures (Table 2-1). Thermal dependence of $\dot{V}_{O_2}$ remained the same with increasing age (Table 2-1). The Q_{10} values between 22-32 °C for embryos at day 16, 30, and 39 were 2.58, 2.28, and 2.48 respectively.

Discussion

Temperature and oxygen consumption

Temperature sensitivity of the metabolic rates of turtle embryos agrees with the study of Zarrow and Pomerat (1937) on the effect of temperature on egg metabolism of the smooth green snake (Opheodrys vernalis). There are numerous studies on the effects of temperature on the embryonic development of reptiles where eggs have been incubated in two or more constant temperature regimes. Eggs that are incubated at warm temperatures grow faster and hatch earlier than those incubated at cool temperatures. These studies have assumed that egg metabolism is temperature-dependent, but this assumption has never been tested extensively. Measurements of snake embryos by Zarrow and Pomerat (1937) and turtle embryos (this study) provide direct evidence for thermal dependence of embryonic metabolism in these two species. The assumption is probably true for species that grow and develop faster in a warm temperature than in a cool temperature simply because rapid

growth and short incubation period most certainly reflect higher rates of metabolism (Ewert 1979; Packard and Packard 1989).

The Q_{10} values of 2-3 measured in red-bellied turtle embryos are rather typical of biological reactions. Similar Q_{10} values for embryos at day 16, 30, and 39 indicate that thermal dependence is similar at different incubation days.

Surprisingly little is known about the thermal dependence of embryonic metabolism in birds. Embryos of domestic fowl have a low Q_{10} of 1.0 for temperatures between 34-40 °C (Greiff 1952), whereas embryos of Heermann's gull (Larus heermanni) have Q_{10}s of 2.05 between 20-30 °C, and 1.0 between 30-40 °C (Bennett and Dawson 1979). The Q_{10} values of avian embryos are therefore generally lower than those of red-bellied turtle embryos, but the reasons are unclear.

The biological significance of a Q_{10} value of 1.0 in Heermann's gull embryos may be that thermal independence within a range of temperature associated with incubation minimizes developmental and physiological disruptions (Bennett and Dawson 1979). The thermal environments of Heermann's gull's nests vary tremendously due to intense solar radiation during the day, and unattended eggs may undergo a wide thermal fluctuations (Bennett and Dawson 1979). Low metabolic sensitivity to acute changes of temperature within certain ranges is commonly found in invertebrates that encounter abrupt changes in their environmental temperature (Newell and Pye 1971; Newell 1979). Compared with Q_{10} values of Heermann's gull embryos, the relatively high Q_{10} of red-bellied turtle embryos indicates that such an adaptation does not occur in this species.

Although metabolic Q_{10} values of reptilian embryos have not been measured before, indirect evidence suggests that Q_{10} values of reptilian

embryos varies interspecifically. Temperature effect on incubation period of turtle embryos was calculated based on the data compiled in Table 4 from Ewert's paper (1979). The incubation period of eggs incubated at 25 and 30 °C was compared. An increase of incubating temperature by 5 °C shortens the incubation period from 2.5 days in Kinosternon flavescens to 86.1 days in Kinosternon scorpioides. Of the 29 species examined, 24 species have incubation periods of 20-40 days shorter at 30 °C than at 25 °C. These interspecific differences in incubation period imply that the thermal dependence of embryonic metabolism is different.

Intraspecifically, populations from different latitudes have different responses to a 5 °C change in incubation temperature. The difference in incubation period between 25 and 30 °C increases as latitude decreases is found in Sternotherus odoratus, Chelydra serpentina, Terrapene carolina, Chrysemys picta, and Pseudemys scripta. In others, the difference remains the same at different latitudes, e.g. in Graptemys pseudogeographica.

The interspecific and latitudinal differences in incubation period in response to an increase of incubating temperature by 5 °C provide excellent opportunities to examine thermal relations of reptilian embryos associated with different environments. For example, if the above observations reflect the differences in thermal dependence, then the Q_{10} of K. flavescens would be very small when compared to that of K. scorpioides, and Q_{10} of northern populations would be smaller than that of southern populations.

Temperature and critical oxygen tension

Any P_c level can be described by an interaction of $\dot{V}_{O_2}$ and critical oxygen gradient, G_c (the slope of the regression line at P_{O_2} below the P_c). If oxygen diffuses across the respiratory surface at the maximum rate at P_{O_2}

below the P_c, then G_c is a measure of the maximum overall oxygen transport capability (Belkin 1965; Beckenbach 1975; Bradford and Seymour 1988). Any enhancement of the circulatory or respiratory capability of an animal that increases oxygen transport will increase the G_c.

At each incubation day, P_c increases directly proportional to temperature (Fig. 2-1 to 2-3 and 2-5), indicating P_c is temperature-dependent. Evidently, acute exposure to temperature changes does not allow time for major structural and biochemical adjustments. In other words, the G_c could not be altered much in a short duration of temperature exposure. Thus, a further increase in metabolism due to elevating temperature results in an increase of P_c (Fig. 2-5). This findings agree with earlier studies on plethodontid salamanders (Beckenbach 1975), but there are no data available to allow comparisons with embryos of other reptiles or birds.

The finding that P_c is temperature-dependent is opposite to earlier studies in fishes where the P_c of most species examined remained constant or decreased with increasing temperature (Ultsch et al. 1978; Ott et al. 1980) . The discrepancy is probably due to differences in experimental protocols. Fishes were well acclimated to test temperatures, whereas plethodontids and turtle embryos were not exposed to test temperatures only at the day of experiment. Chronic exposure to a warm temperature could induce physiological changes such as ventilation, heart rate, and hematocrit to facilitate oxygen transport (Spitzer et al. 1968).

Reptilian and avian embryos obtain air through eggshell pores or spaces by diffusion. Depending upon the resistances of diffusion pathways, the diffusion rate of a gas could vary among or within species (Carey et al. 1982). At each day of incubation, embryos are able to maintain resting metabolism when the ambient P_{O2} is as low as 110-120 mmHg. In contrast,

Temple and Metcalfe (1969) and Ar et al. (1991) reported that the gas exchange of avian eggs is near maximum under normoxic conditions. Embryos of 16 days could not maintain resting metabolism when subjected to acute hypoxia (below 159 mmHg), indicating that they are metabolic conformers (Ar. et al. 1991). What happens to embryonic metabolism if the egg temperature is elevated above normal? Because avian embryos operate at a maximum rate of gas diffusion under normal conditions, the additional oxygen demand due to increased temperature could probably not be achieved. This would not produce a change in oxygen consumption, which might offer an alternative explanation for why embryos of Heermann's gull have a Q_{10} of 1 at 30-40 °C (Bennett and Dawson 1979).

Development and critical oxygen tension

Any changes in P_c during development result from dynamic interaction between $\dot{V}_{O_2}$ and G_c. Embryonic development increases $\dot{V}_{O_2}$, which increases the P_c. Conversely, development of circulatory and respiratory systems promotes oxygen transport and reduces the P_c by changing the G_c.

The effect of incubation day on P_c varies among temperatures. At 27 °C, the P_c increases as embryos develop, which is similar to findings in an earlier study (see chapter 1). However, the P_c of embryos at 32 °C is maintained high at between 110 and 117 mmHg, and does not change much during incubation. It is surprising to find that the P_c of embryos at day 16 is 116 mmHg, while an earlier study showed that values for P_c on days 12 and 26 at 30 °C were only 75 and 86 mmHg respectively (see chapter 1). Two possible reasons might account for the high P_c at day 16. Firstly, the chorioallantoic membrane has not developed completely, and oxygen transport capacity is not as high as that in older embryosis. Secondly, embryos do not function normally at such high

temperatures. For most embryonic turtles, 32 °C represents a high limit of thermal tolerance (Packard and Packard 1989). In bird embryos the tolerance to acute heat exposure is low at early development (Moreng and Shaffner 1951; MacMullan and Eberhart 1953; Romanoff 1960).

At 22 °C, the P_c of embryos was low, and it decreased at the older age (Fig. 2-7). As embryos grew, the oxygen transport system, including chorioallantoic membrane and circulatory system, developed to meet increasing oxygen demand. Turtle eggs in this study were incubated at constant 30 °C, so at any given time, the oxygen transport system developed in response to the metabolic demand of embryos at 30 °C. However, when embryos were acclimated at 22 °C, oxygen consumption decreased and the metabolic demand was low. As a result, embryos at 22 °C could maintain resting metabolism at even lower hypoxic conditions. Similarly, ectothermic animals behaviorally lower body temperature when exposed to hypoxia, thereby lowering the metabolic demand and enabling the animals to maintain resting metabolism at more severe hypoxic conditions (Hicks and Wood 1985; Wood et al. 1987).

Ecological considerations

Results of this study have shown that turtle embryos are hypoxia-tolerant organisms, and their hypoxic tolerance decreases with increased temperature (Table 2-1). Thus, it appears to be advantageous to bury eggs deep in the nest mounds of alligators. This is because the location of the turtle nest cavities in alligator nest mounds is a compromise between hypoxia and temperature. A shallow nest in the upper part of a nest mound experiences little or no hypoxia but a wide range of temperature. The situation is just the opposite for a nest located deeper inside a nest mound.

This speculation is supported by an earlier observation that eggs are usually located either level with or below the alligator nest cavities (Goodwin and Marion 1977). Alligator nest cavities have temperatures that vary only 1.2 °C daily, while the mean hourly air temperature fluctuates 9.3 °C daily (Chabreck 1973). Thus, by putting eggs at the vicinity of alligator nest cavities, turtle embryos experience a rather stable thermal environment.

Table 2-1. Oxyen consumption ($\dot{V}_{O_2}$) and critical oxygen tension (P_c) at different temperatures and incubation times. Values are reported as means with standard error in parentheses. Q_{10} is calculated for each successive increase in temperature. Temp = temperature; N = sample size; W = egg mass.

	Temp (°C)	N	W (g)	$\dot{V}_{O2}$ (ml / h)	P_c (mmHg)	Q_{10}
DAY 16	22	5	13.10 (0.11)	0.092 (0.004)	62.5 (3.5)	
						2.98
	27	5	12.86 (0.27)	0.159 (0.006)	79.6 (3.2)	
						2.22
	32	5	13.31 (0.32)	0.237 (0.003)	116.4 (2.3)	
DAY 30	22	4	13.81 (0.40)	0.534 (0.021)	60.7 (1.9)	
						2.36
	27	4	13.79 (0.18)	0.821 (0.043)	87.0 (1.7)	
						2.20
	32	4	14.03 (0.31)	1.217 (0.028)	109.4 (5.0)	
DAY 39	22	4	13.49 (0.15)	0.763 (0.008)	47.7 (3.3)	
						2.85
	27	4	12.87 (0.38)	1.289 (0.039)	99.8 (10.0)	
						2.15
	32	4	13.35 (0.26)	1.891 (0.011)	117.0 (6.6)	

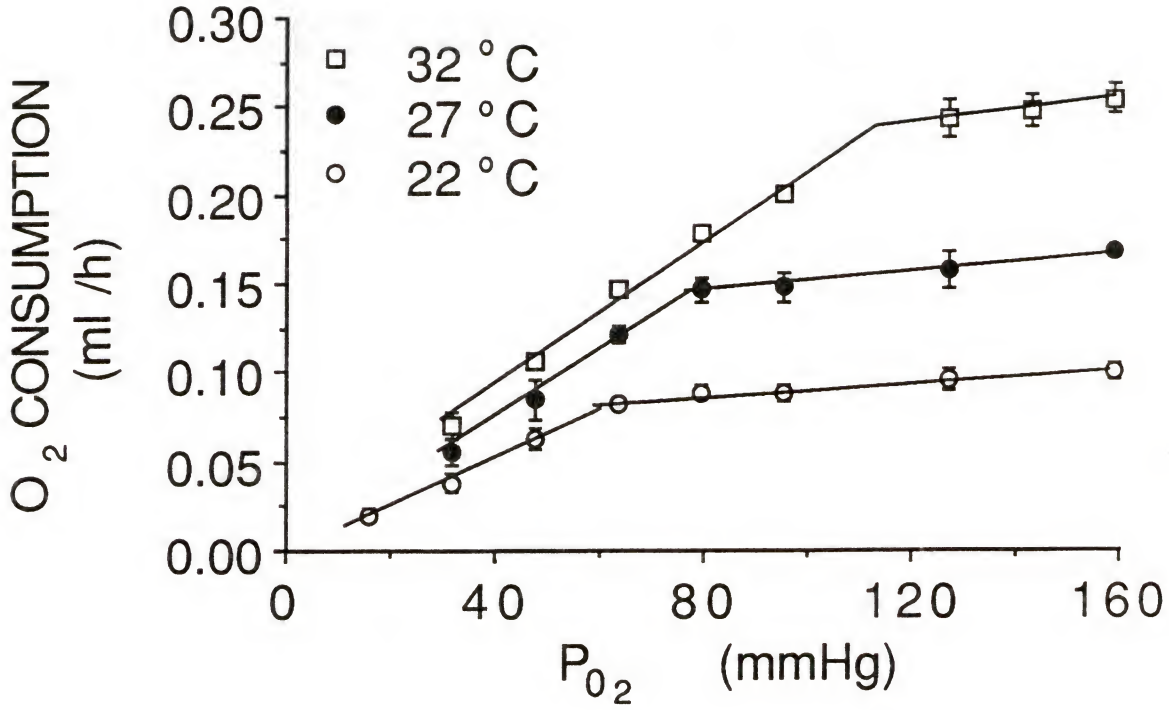

Figure 2-1. Oxygen consumption (ml / h) of embryos at day 16 as a function of oxygen tension at different temperatures. Values are means ± SE.

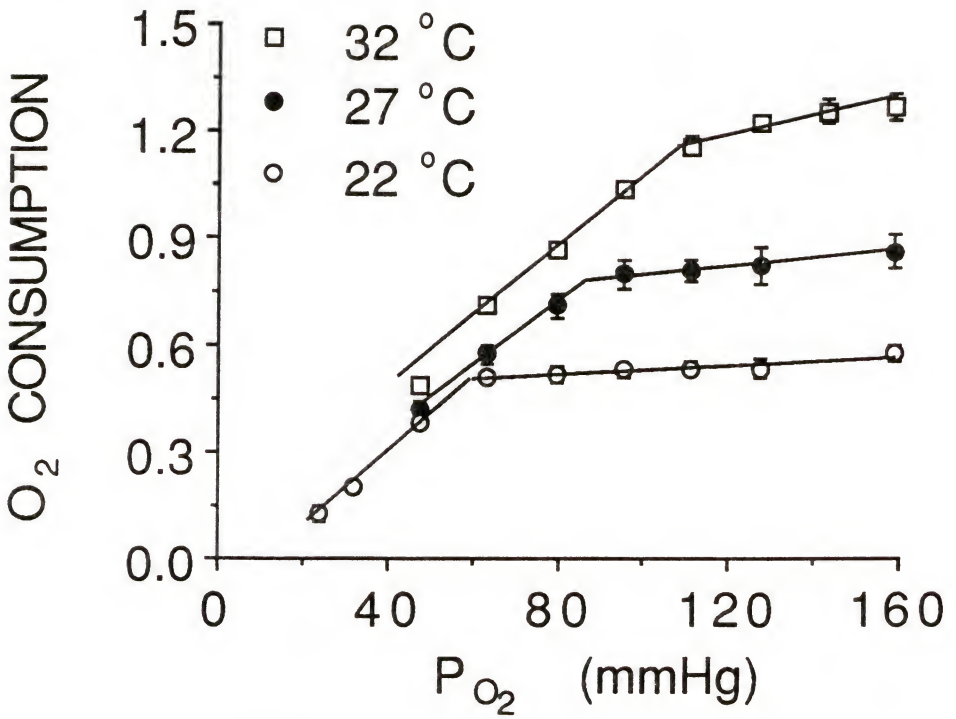

Figure 2-2. Oxygen consumption (ml / h) of embryos at day 30 as a function of oxygen tension at different temperatures. Values are means ± SE.

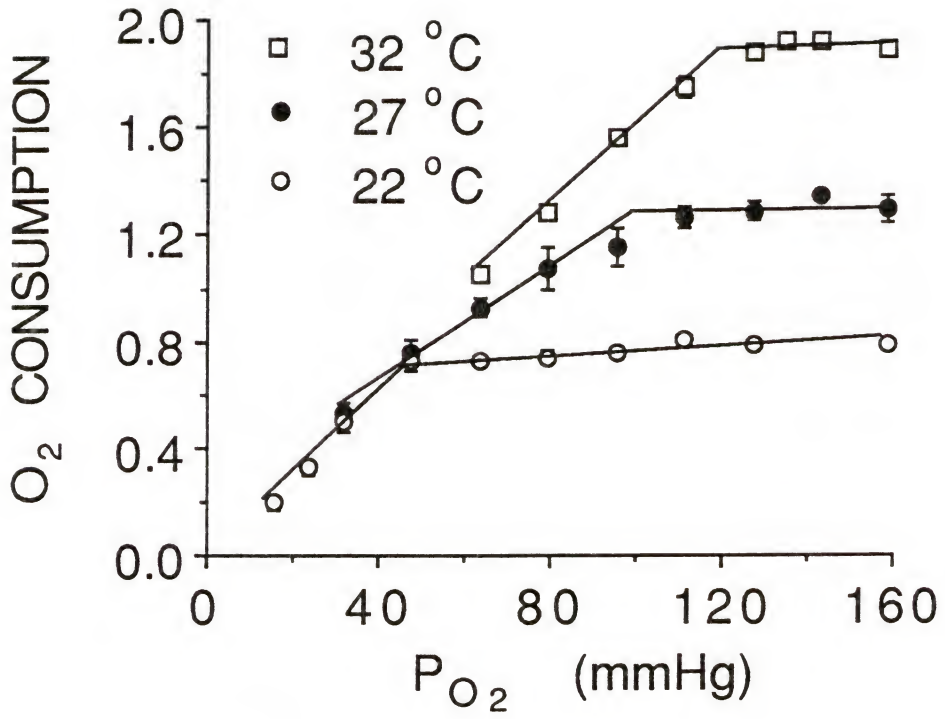

Figure 2-3. Oxygen consumption (ml / h) of embryos at day 39 as a function of oxygen tension at different temperatures. Values are means ± SE.

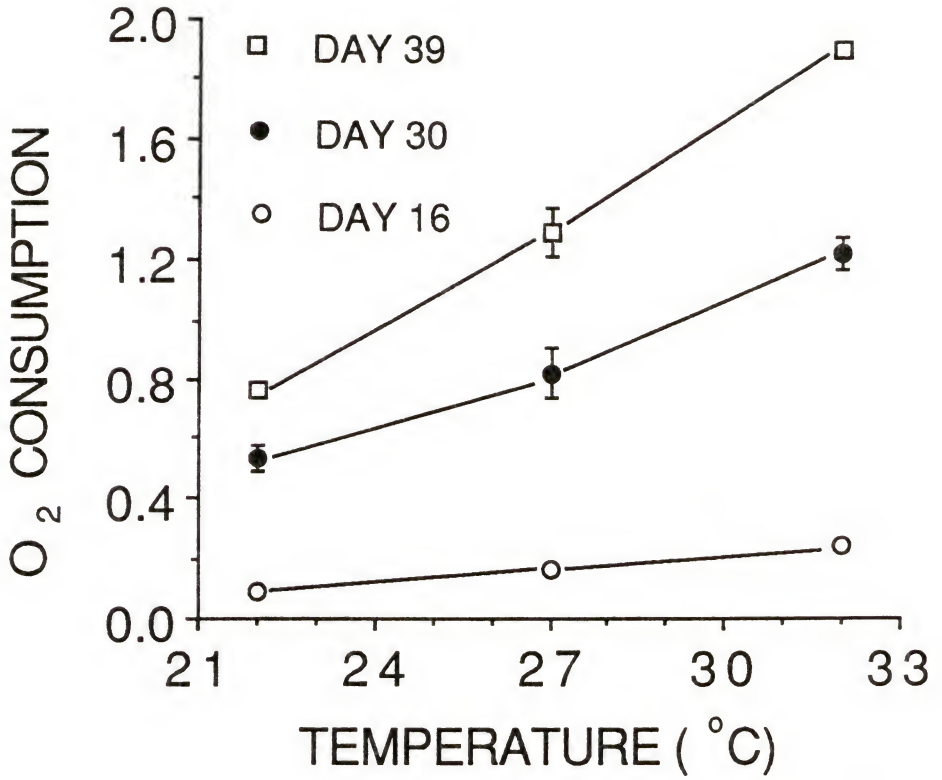

Figure 2-4. Oxygen consumption (ml / h) of eggs as a function of temperature at different incubation days. Values are means ± SE.

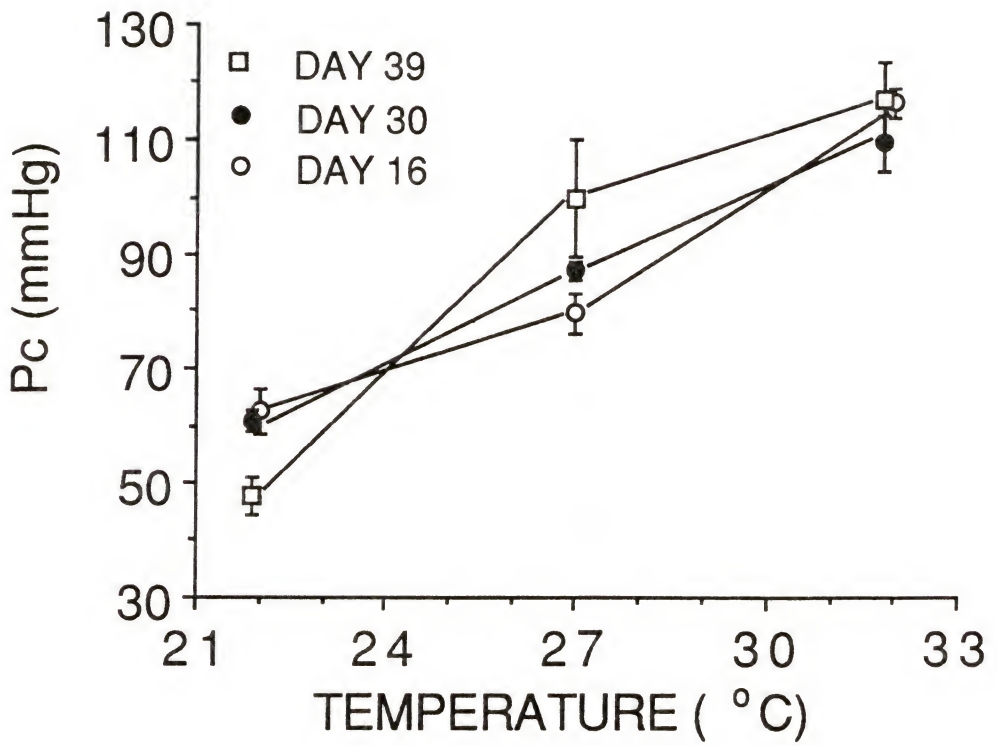

Figure 2-5. Relationship of the critical oxygen tension (P_c) of embryos to temperature at different incubation days. Values are means ± SE.

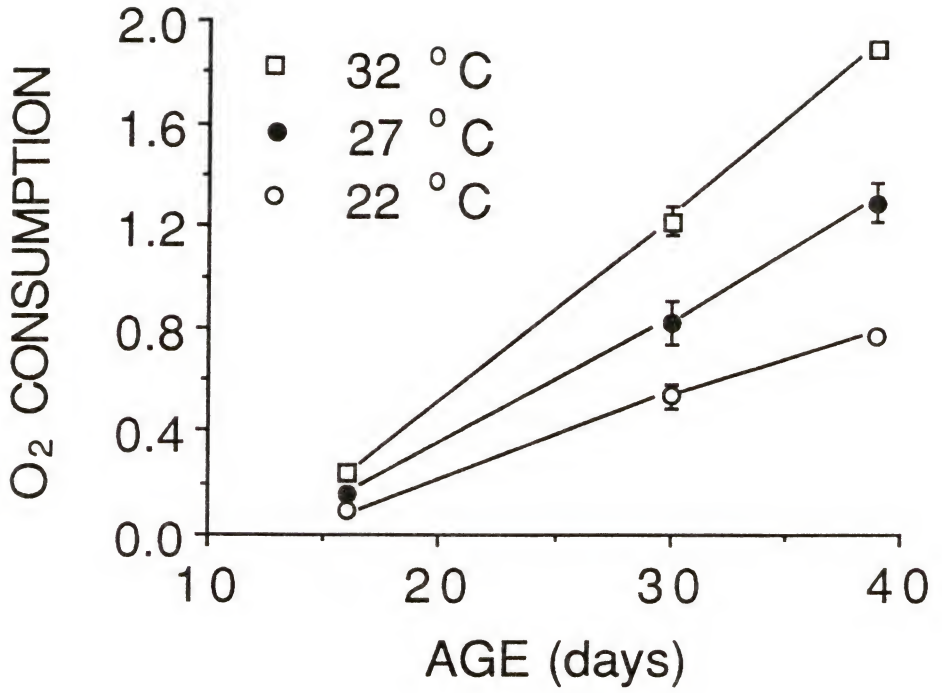

Figure 2-6. Oxygen consumption (ml / h) of eggs as a function of incubation days at different temperatures. Values are means ± SE.

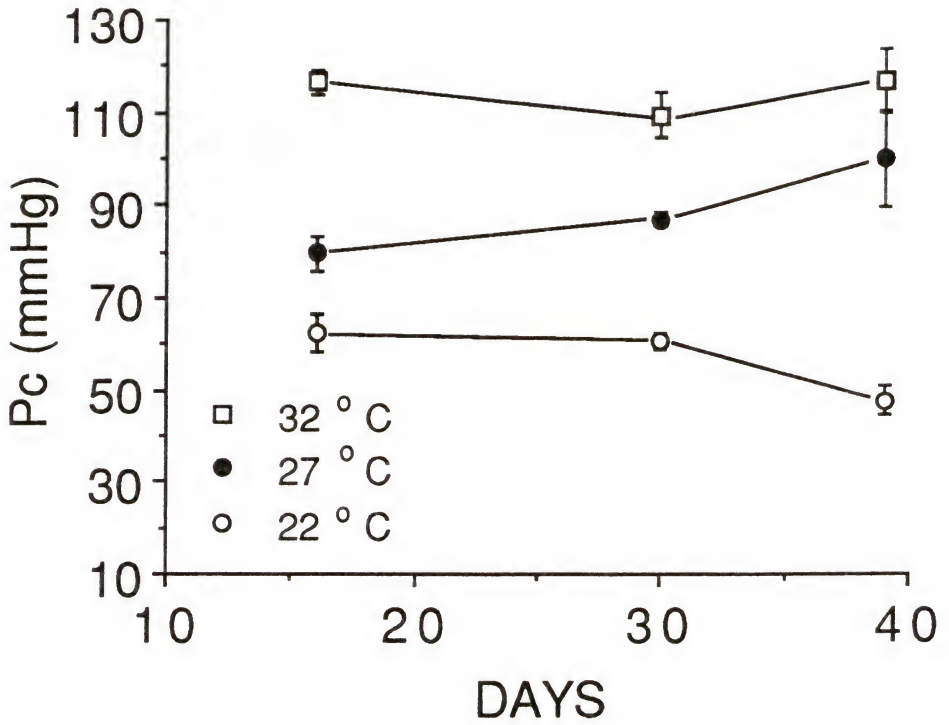

Figure 2-7. Relationship between the critical oxygen tension (P_c) of embryos and incubation day at different temperatures. Values are means ± SE.

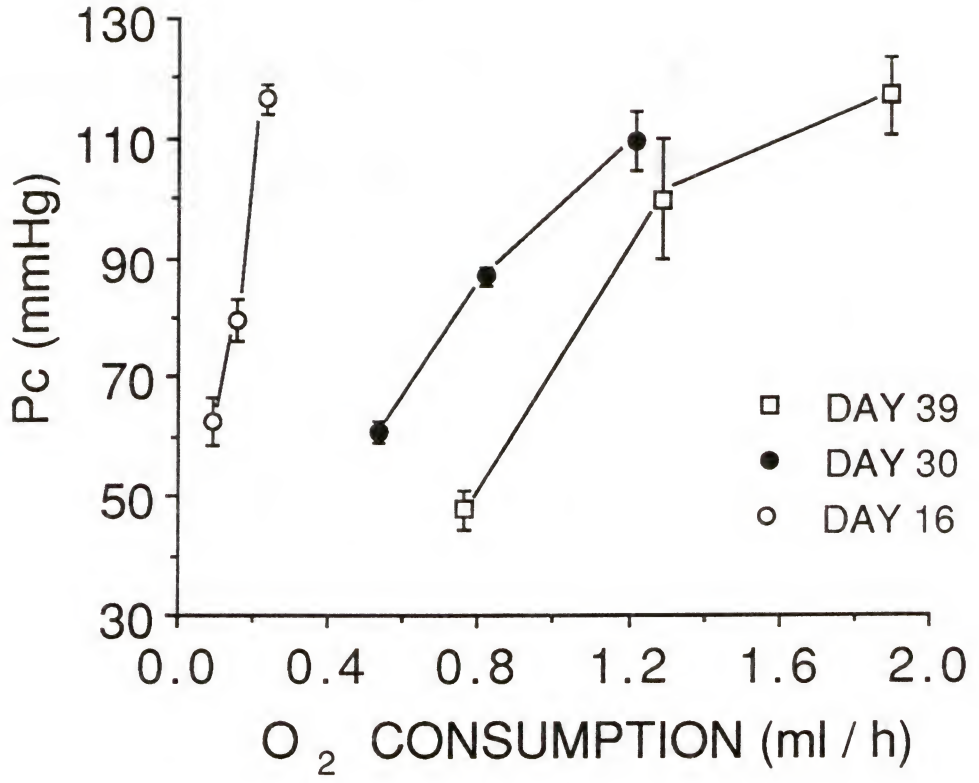

Figure 2-8. Relationship between critical oxygen tension (P_c) and oxygen consumption of Florida red-bellied turtle embryos. Values are means ± SE.

CHAPTER 3
INFLUENCES OF EGG HYDRATION ON METABOLISM, CRITICAL OXYGEN TENSION, AND HATCHLINGS OF FLORIDA RED-BELLIED TURTLE (PSEUDEMYS NELSONI)

Introduction

Numerous studies have demonstrated that flexible-shelled reptilian eggs take up water from their surroundings under favorable conditions, and the amount of water uptake depends on the water potential and thermal conductivity of the incubation media (Packard et al. 1977; Packard and Packard 1984;1989; Kam and Ackerman 1990). However, whether this water uptake process affects gas exchange of egg is not clear. Even though water and oxygen move across the eggshell-membrane complex by different pathways, the oxygen conductance of eggs is still hydration-dependent (Ackerman and Prange 1972; Lutz et al. 1980; Feder et al. 1982; Black et al. 1984). For example, in flexible eggs of the snapping turtle (Chelydra serpentina), membranes contribute the majority of the resistance to oxygen diffusion. Oxygen conductance of moist eggshell-membrane complexes falls 10-fold when compared to that of dry eggshell-membrane complexes (Feder et al. 1982). Black et al. (1984) incubated Burmese python (Python molurus bioittatus) eggs in different moisture regimes, and they found that the oxygen conductance of the eggshell-membrane complex is inversely correlated with the amount of water uptake.

The reduction in oxygen conductance may not affect growth and development of embryos if eggs are incubated under normoxic conditions, but it

could become a problem when eggs are incubated under hypoxic conditions. Eggs incubated underground are potentially subjected to hypoxia when oxygen concentration in the nest chambers is reduced. This reduction in oxygen can be caused by a combination of a low gas conductance of the nest due to the wetting of incubation medium by rainfall, flooding, and high tides (Chabreck 1975; Plummer 1976), microbial respiration (Seymour et al. 1986), and egg metabolism (Ackerman 1977; Lutz and Dunbar-Cooper 1984).

Flexible-shelled eggs of the Florida red-bellied turtle, Pseudemys nelsoni were used to investigate the effects of water relations of eggs on embryonic metabolism and growth and respiration under hypoxic conditions. Critical oxygen tension measurements were used to evaluate how well embryos tolerate hypoxia.

Materials and Methods

A total of five clutches of Florida red-bellied turtle eggs was collected from alligator nests in Florida during June and July, 1991. One egg from each clutch was opened to determine age. Eggs from each clutch were assigned to both moisture regimes, 3 and 13 % gravimetric water content, to eliminate clutch effects. A total of 38 eggs was assigned to each group, and they were half-buried in sand and incubated at a constant 30 °C. Boxes were covered with plastic sheets to reduce evaporative water loss, and water was added as necessary.

Mass and critical oxygen tension were measured on six randomly selected eggs from each group on day 16, 30, and 39. The critical oxygen tension was determined by a method described elsewhere (see chapter 1).

Briefly, each egg was placed in a cup inside a metabolic chamber containing 5 ml of distilled water. The metabolic chambers were submerged in a water bath at 32 º C for 3 hours before measurements were taken. Chambers were flushed and reflushed with a humidified gas mixture before stopcocks were closed. A 30 ml gas sample was collected from each chamber at the end of each measurement, and the final oxygen fraction was determined by injecting the sample into an Applied Electro-chemistry S-3A O_2 analyzer. Immediately after the O_2 concentration of all chambers was measured, eggs were subjected to the next gas mixture, ranging from 90 to 10% air, and the oxygen concentrations were determined by repeating the above protocol. Each new gas mixture was 5 to 20 % lower in air content than the previous treatment. Oxygen consumption, $\dot{V}_{O_2}$ (ml. STDP / h egg), was calculated using the equation, $\dot{V}_{O_2} = V(FI_{O_2}-FE_{O_2})/(1-FE_{O_2})t$, where V is the dry, CO_2-free air (ml), FI_{O_2} and FE_{O_2} are the initial and final oxygen fraction (%) of a measurement, and t is the duration (h) from start to end of a measurement (Vleck 1987). The critical oxygen tension, P_c (the P_{O_2} at which the oxygen consumption is first reduced), was determined using a BASIC program that fits a two-segmented straight line (Yeager and Ultsch 1989). Data were analyzed individually and averaged.

Wire partitions were installed when pipped eggs were found in any box. Eggs were checked daily, and hatchlings were quickly frozen in air-tight containers. They were later thawed at room temperature, and residual yolk and carcass were separated, weighed, dried at 65 ºC, and reweighed. Egg mass, hatchling mass, and oxygen consumption were adjusted using analysis of covariance (ANCOVA) and the initial mass was used as covariate. All means were expressed as means (± standard error) unless otherwise noted. In most cases, means were tested for significant difference using Student's t test.

Results

Eggs in both groups increased mass throughout incubation, and the patterns of water uptake were similar (Fig. 3-1). Eggs incubated in 3 % gravimetric water content increased mass slowly and gained 0.67 and 1.59 g of water by day 30 and 39 respectively (Fig. 3-1). In contrast, eggs incubated in 13 % gravimetric water content increased mass rapidly and gained 1.83 and 3.47 g of water by day 30 and 39, respectively (Fig. 3-1). Egg masses between two groups were different at day 30 and 39 ($P < 0.05$ and $P < 0.01$ respectively).

Critical oxygen tension curves of embryos for both groups at day 16, 30, and 39 are presented in Figures 3-2, 3-3, and 3-4 respectively. Oxygen consumption of embryos were identical between two groups at day 16, 30, and 39. Figures 3-5 and 3-6, drawn from Figures 3-2 to 3-4, illustrate the changes of oxygen consumptions and critical oxygen tensions during incubation. Oxygen consumption measured in air were used in this comparison. The results show embryos consumed more oxygen as they grew, and the rates of increase in the two groups were almost identical (Fig. 3-5). The critical oxygen tensions remained unchanged over the incubation period and were not different between groups (Fig. 3-6). The dry masses of yolk-free hatchlings were the same between groups. Eggs incubated in 13 % gravimetric water content, however, had heavier wet mass of hatchlings and less yolk, wet and dry, than eggs incubated in 3 % gravimetric water content (Table 3-1).

Discussion

The similarity of oxygen consumption and dry mass of yolk-free hatchlings in the two groups suggests that growth and development are not affected by the moisture levels of sand (Fig. 3-5; Table 3-1). A similar finding has been reported for Burmese python eggs (Black et al. 1984). Eggs incubated in different moisture regimes had similar oxygen consumption and hatchling mass, while O_2 conductance is inversely correlated to the moisture levels (Black et al. 1984). Because metabolism and growth are not affected by the moisture regimes, embryos incubated in moist substrates must have maintained normal metabolism and growth by extracting more oxygen from blood, which reduces the P_{O2} beneath the eggshell (Black et al. 1984).

However, as mentioned earlier, the low P_{O2} beneath the shell may have detrimental effects on metabolism when eggs are exposed to hypoxia. The transport of oxygen from atmospheric air to cells involves three major compartments: the gas exchanger (the chorioallantoic membrane); the oxygen carrier (blood); and the oxygen sink (cells) (Weibel 1984). These compartments are in a series arrangement, and P_{O2} drops as oxygen moves from one compartment to the other. Note that two of the three compartments require diffusion and are driven by a P_{O2} gradient. Any factor that increases P_{O2} at any point could facilitate oxygen transport, whereas any factor that decreases P_{O2} at any point could impede oxygen transport. In theory, Burmese python eggs incubated in the moist regimes should have a lower P_{O2} under the eggshell and are predicted to be less able to maintain normal oxygen consumption under hypoxic conditions than eggs incubated in the dry regimes.

Results in this study disagree with the prediction, as indicated by the similarity of critical oxygen tensions (day 30 and 39) even though eggs in 13 %

gravimetric water content gained significantly more water than those in 3 % gravimetric water content. Two explanations could account for the differences. 1) Turtle eggs may have a higher O_2 conductance and, therefore, the P_{O2} gradient across the eggshell-membrane complex are smaller than in Burmese python eggs. Consequently, the reduction in O_2 conductance due to moisture treatments in this study would not be severe enough to cripple the growth and development of embryos. 2) Black et al. (1984) measured the P_{O2} beneath the shell when eggs were half-buried in vermiculite. Thus, the total resistance to oxygen diffusion is the sum of the resistance of substrate and shell-membrane complex. In contrast, critical oxygen tension of embryos was measured outside the incubating substrates, and the total resistance to oxygen diffusion was attributable only to the shell-membrane complex and presumably smaller than measurements made by Black et al. (1984).

In conclusion, for reptilian eggs incubated underground in natural environments, the incubating media may affect gas exchange of eggs directly and indirectly. As the medium becomes wet, the resistance to oxygen diffusion increases (Kam 1988; McGehee 1990). In addition, eggs take up more water when incubated in moist conditions, and the increased water content in the eggshell-membrane complex also increases the resistance to oxygen diffusion (Black et al. 1984). However, the egg shell may crack as eggs take up water and swell, which presumably increases O_2 conductance (Lillywhite and Ackerman 1984). Depending on the amount of water uptake, shell type, and the severity of hypoxia inside nest chambers, the sum of these two resistances could be high enough to impede oxygen diffusion and retard embryonic growth.

Table 3-1. Hatchling mass, residual yolk mass, and incubation period of Florida red-bellied turtle eggs incubated in 3 and 13 % gravimetric water content. Values are reported as means with standard error in parenthesis. P is significant probability.

	3 %	13 %	P
Sample size	14	14	
Incubation time (days)	43 (0.3)	44 (0.4)	> .05
Wet hatchling mass (g)	7.37 (0.09)	7.92 (0.09)	< .001
Dry hatchling mass (g)	1.76 (0.05)	1.88 (0.05)	> .05
Wet residual yolk mass (g)	1.65 (0.08)	1.41 (0.08)	< .05
Dry residual yolk mass (g)	0.82 (0.04)	0.66 (0.04)	< .01

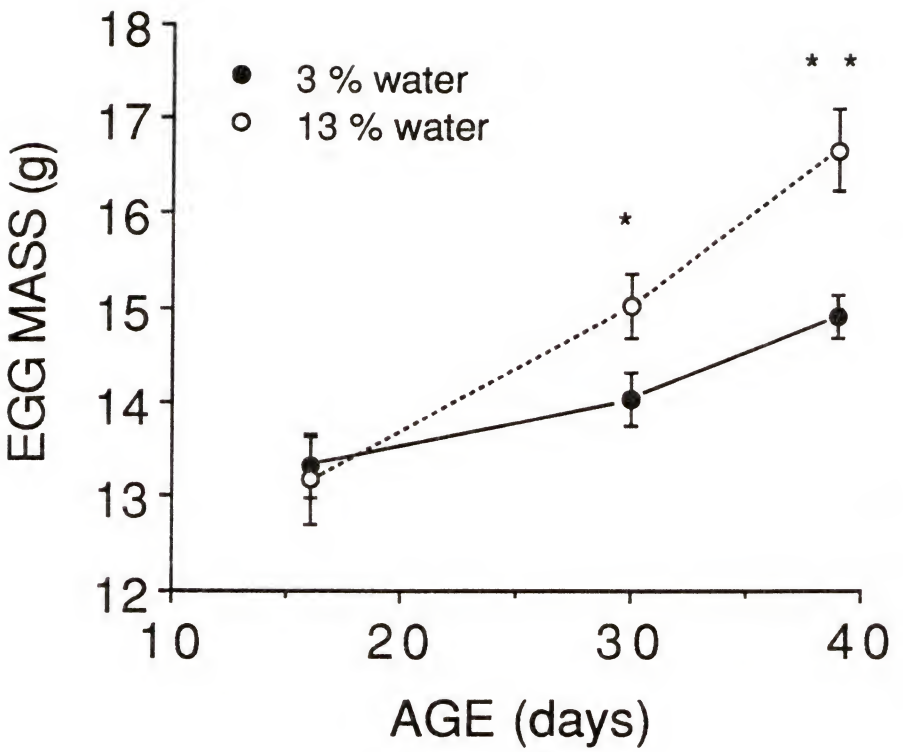

Figure 3-1. Mean values (±SE) for changes in mass of eggs incubated in sand with 3 (solid line) and 13 % (dashed line) gravimetric water content. * and ** represent significant differences at 0.05 and 0.01 levels, respectively.

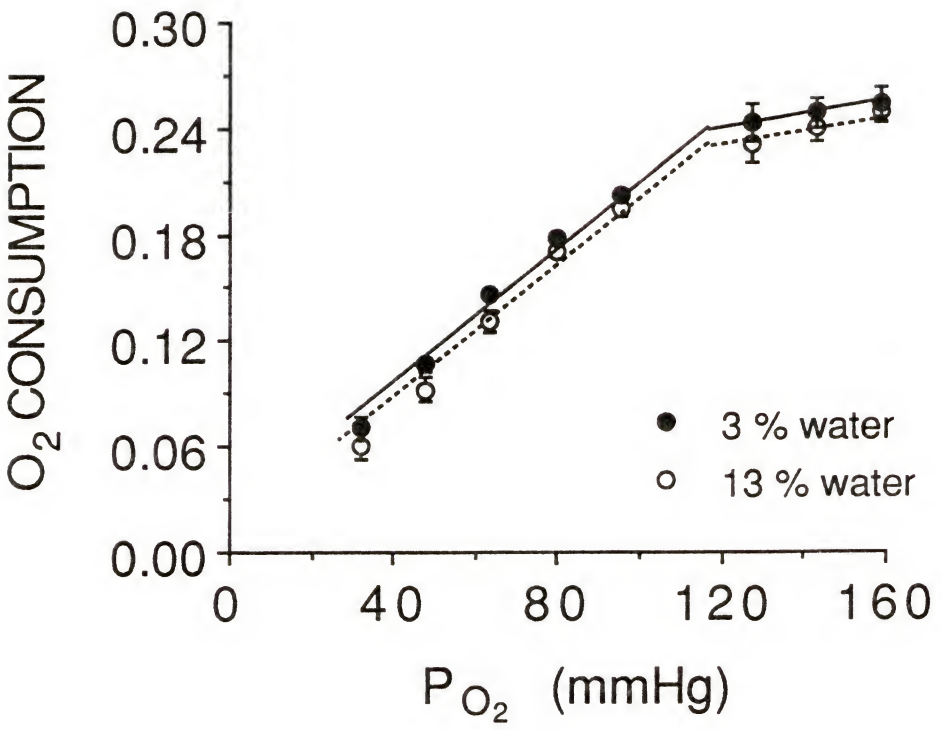

Figure 3-2. Oxygen consumption (ml / h) of embryos at day 16 as a function of oxygen tension. Eggs were incubated in sand with 3 (solid line) and 13 % (dashed line) gravimetric water content. Values are means ± SE.

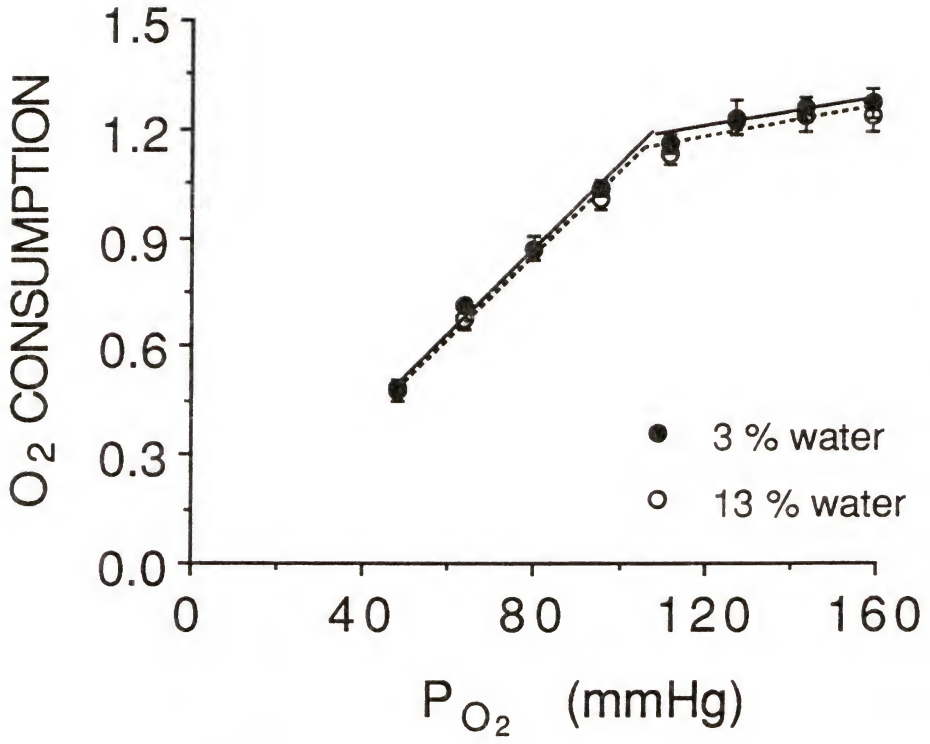

Figure 3-3. Oxygen consumption (ml / h) of embryos at day 30 as a function of oxygen tension. Eggs were incubated in sand with 3 (solid line) and 13 % (dashed line) gravimetric water content. Values are means ± SE.

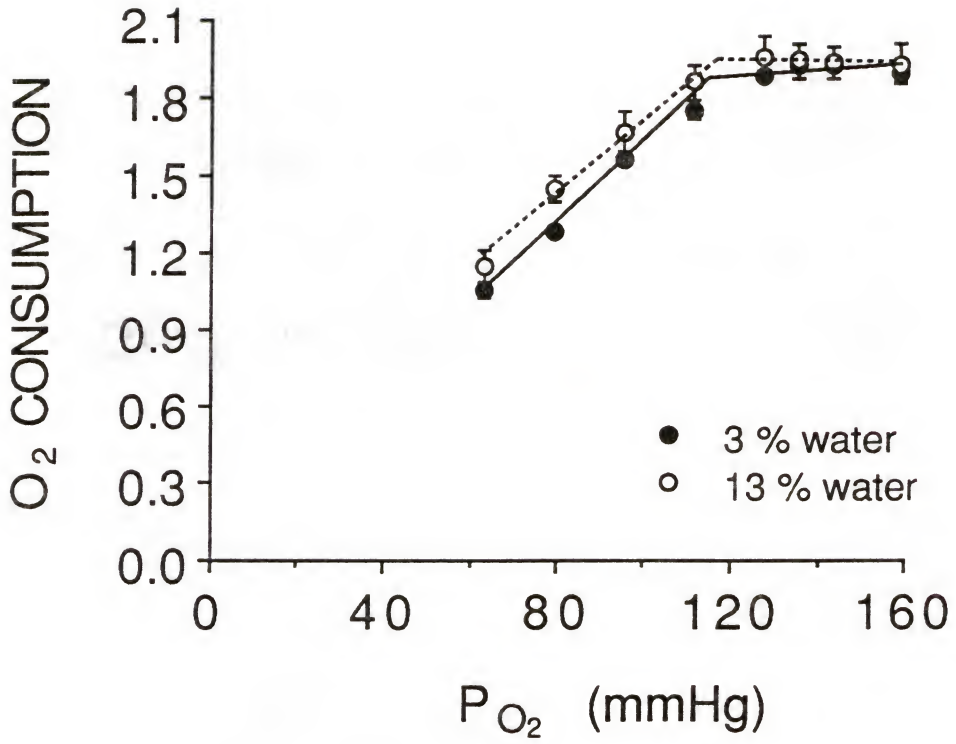

Figure 3-4. Oxygen consumption (ml / h) of embryos at day 39 as a function of oxygen tension. Eggs were incubated in sand with 3 (solid line) and 13 % (dashed line) gravimetric water content. Values are means ± SE.

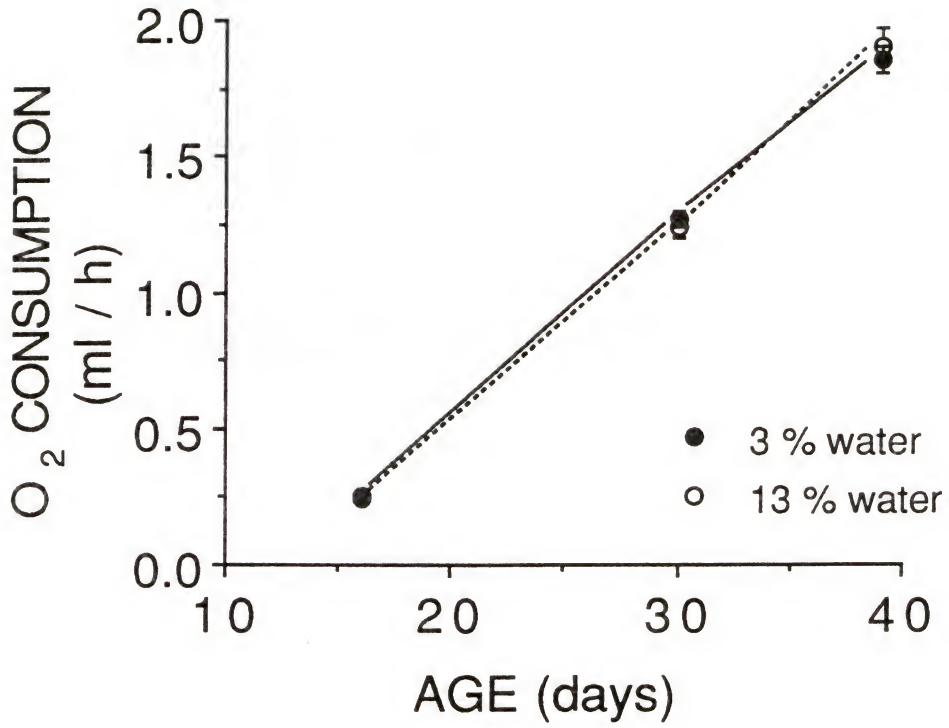

Figure 3-5. Oxygen consumption of embryos incubated in sand with 3 (solid line) and 13 % (dashed line) gravimetric water content. Values are reported as means ±SE.

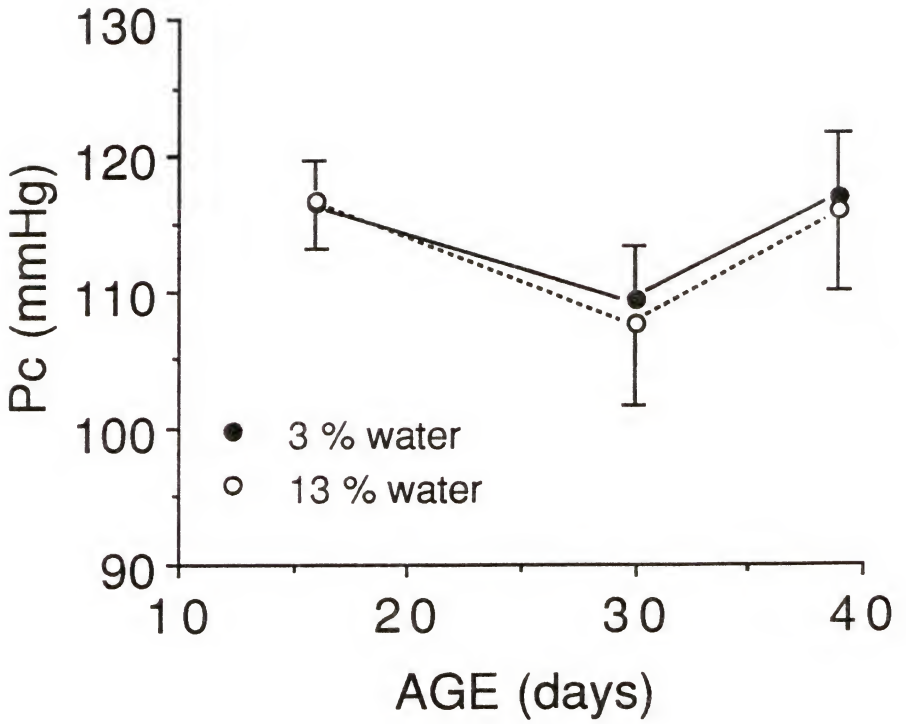

Figure 3-6. Relationship between the critical oxygen tension (P_c) of embryos and incubation day. Eggs were incubated in sand with 3 (solid line) and 13 % (dashed line) gravimetric water content. Values are means $\pm$ SE.

CHAPTER 4
PHYSIOLOGICAL RESPONSES OF FLORIDA RED-BELLIED TURTLE (PSEUDEMYS NELSONI) EMBRYOS TO CHRONIC HYPOXIA

Introduction

Reptilian eggs incubated underground are potentially subjected to hypoxia when oxygen concentration in nest chambers is reduced. Reduction in oxygen can be caused by a combination of a low gas conductance of the nest due to the wetting of incubation medium by rainfall, flooding, or high tides (Chabreck 1975; Plummer 1976), microbial respiration (Seymour et al. 1986), and egg metabolism (Ackerman 1977; Lutz and Dunbar-Cooper 1984).

Hypoxic effects on embryonic metabolism and growth of avian eggs have received far more attention than in reptilian eggs. Avian embryos generally tolerate moderate hypoxia (P_{O_2} = 125 mmHg) where development and growth are not affected (Black and Snyder 1980; Synder et al. 1982; Carey et al. 1982). Severe hypoxia (P_{O_2} = 95 mmHg or lower) retards development and growth and prolongs the incubation period in chicken and montane coot (Fulica americana) embryos (Wangesteen et al. 1974; Carey et al. 1989). In most cases, the development and growth of high altitude species are not affected by hypoxia encountered in natural habitats, as found in bar-headed geese (Anser indicus), horned larks (Eremophila alpestris), and red-winged blackbirds (Agelaius phoeniceus) (Carey et al. 1982; Snyder et al. 1982; Verbeek 1967; Conry 1978). These studies and others have also shown that exposure to hypoxia induces structural and physiological adjustments of embryos in

respiratory, circulatory, and tissue levels (Black and Synder 1980; Carey et al. 1982; Baumann et al. 1983; Dusseau and Hutchins 1988; Strick et al. 1991)

Hypoxic effects on embryonic growth and development of reptiles have been studied only in sea turtle (Ackerman 1981a) and snapping turtle (Chelydra serpentina) embryos (Kam 1988). Green turtle (Chelonia mydas) and loggerhead (Caretta caretta) eggs were incubated in artificial nests where the gas conductance of the sand could be manipulated. Ackerman (1981a) found that eggs incubated in nests under a long sand column grow slower and hatch later than those incubated in nests under a short sand column. Kam (1988) reported that embryos incubated in sand with 12 % gravimetric water content grew more slowly than those with 4 % gravimetric water content. McGehee (1990) incubated loggerhead eggs in sand with 0, 25, 50, 75, and 100 percent moistures, and found that the hatching success is strongly related to the moisture levels. These findings suggest that retarded metabolism and growth of embryos are due to oxygen depletion and possibly carbon dioxide accumulation in nests, which are caused by a low gas conductance in the incubation medium, attributable to either sand thickness or moisture. Oxygen and carbon dioxide tensions in nest chambers during incubation were not reported or measured. Therefore, it is not known whether embryos could tolerate hypoxia or could compensate for hypoxia and hypercapnia.

The hypoxic effects on the embryonic growth and development of Florida red-bellied turtle (Pseudemys nelsoni) embryos were studied. Many Florida red-bellied turtles found in wetlands of north-central Florida lay their eggs in nest mounds of the American alligator (Alligator mississippiensis) (Goodwin and Marion 1977). Oxygen content within a nest cavity is dependent upon the nest location and rainfall during the nesting season. In this study, hypoxic tolerance was determined by measuring the critical oxygen tension of embryos.

Morphometries of blood vessels in the chorioallantoic membrane, heart mass, and hematocrit were measured to reveal possible responses to hypoxia.

Materials and Methods

A total of ten clutches of Florida red-bellied turtle eggs were collected from alligator nests in Alachua county, Florida during June and July, 1991. One egg from each clutch was opened to determine age. Eggs were randomly and equally assigned to a control and a hypoxic treatment. Both group of 77 eggs initially were half-buried in sand with 4 % gravimetric water content and incubated at a constant 30 °C. The boxes were covered with plastic sheets to reduce evaporative water loss, and water was added as necessary.

At 21 days of incubation, eggs were transferred to sealed incubators (dimension 45 x 30 x 25 cm) and half-buried in sand with 4 % gravimetric water content. Two outlets, one for incurrent and one for outcurrent gas, were installed on the top cover. Gas was humidified by bubbling through a gas-sealed flask containing distilled water before being delivered into the incubators. One incubator was perfused with air delivered by an air pump located upstream, and the other incubator was perfused with a mixture of 90 % N_2 and 10 % O_2 from a compressed gas tank. Flow rates were set at about 4-5 L / h. The oxygen tensions in both incubators were checked daily by taking a gas sample and measured with an Applied Electro-chemistry S-3A O_2 analyzer. They averaged 150 $\pm$ 1 and 72 $\pm$ 5 mmHg for the normoxic and hypoxic incubators, respectively. Relative humidity inside the incubators was high throughout incubation, as indicated by the condensation of water vapor on the

walls. Eggs in both incubators had positive water balance throughout incubation, which increased 5-10 % of their intial mass.

Mass and oxygen consumption were measured from 6-12 randomly selected eggs from each incubator on day 21, 26, 33, and 39. Eggs were then opened. Yolk and embryo were separated, weighed, dried at 65 °C, and reweighed.

Wire partitions were installed when pipped eggs were found in any incubator. Eggs were checked daily, and hatchlings were killed with halothane. Blood was collected from a carotid artery to determine hematocrit, and hatchlings were frozen in air-tight containers. Later, residual yolk, ventricle, and carcass were separated, weighed, dried at 65 °C, and reweighed.

The oxygen consumption of eggs was measured using a closed-system, and Glass jars (Kerr Glass Inc.) fitted with two rubber stoppers and three-way stopcocks were used as metabolic chambers. These chambers contained 5 ml of distilled water and a piece of wet filter paper to keep the chambers at 100 % relative humidity. Each chamber was flushed with humid air before stopcocks were closed. The interval for gas sampling was dependent on the developmental stage of embryos such that no more than 1-2 % of total oxygen was consumed. A 40 ml gas sample was taken at the end of incubation, and the fractional O_2 concentration was determined with an Applied Electrochemistry S-3A O_2 analyzer. Oxygen consumption, $\dot{V}_{O_2}$ (ml O_2. STDP / h egg), was calculated using the equation, $\dot{V}_{O_2} = V(FI_{O_2}-FE_{O_2})/(1-FE_{O_2})t$, where V is the dry, CO_2-free air, FI_{O_2} and FE_{O_2} are the initial and final oxygen fractions (%) of a measurement, and t is the duration (h) from start to end of a measurement (Vleck 1987).

On day 21 and 33 of incubation, six eggs from each group were selected to measure critical oxygen tensions. Methods used to measure the oxygen

consumption of embryos at different gas tensions and to determine the critical oxygen tension are described earlier (Chapter 1). After the experiments, eggs were fixed in 10 % buffered formalin for 1-3 days. Eggs were later opened, the chorioallantoic membrane (CAM) was removed and fixed, and embryo and yolk were separated, dried at 65 ^{o}C, and weighed.

The morphometry of CAM (surface area and length of blood vessels) was quantified using a digitizer adjacent to a Nikon Labophot microscope. Raw data were stored in an Apple IIe microcomputer interfaced with the digitizer. A vascular density index was also determined by counting the number of vessels that intersected with the lines of a grid. Measurements were done at 40 X magnification, and all counting was done in triplicate and averaged.

Hatchling mass and oxygen consumption were adjusted using analysis of covariance (ANCOVA) and the initial mass was used as covariate. The mass and oxygen consumption of embryos from the critical oxygen tension experiments were not different from that of the other experiment, and therefore, the data were pooled. All means were expressed as means (SE) unless otherwise noted.

Results

Eggs in control and hypoxic groups increased oxygen consumption during incubation and reached 1.38 and 1.36 ml O_2/ h, respectively, at day 39 (Fig. 4-1). Oxygen consumption of eggs in the normoxic conditions reached a plateau at day 33, whereas that of eggs in hypoxic conditions did not reach the plateau until day 39. The oxygen consumption of eggs in both groups was different at day 33.

Embryos in normoxic and hypoxic conditions grew at an increasing rate (Fig. 4-2). Dry embryonic masses were not different between groups until day 39 (control group with 1.18 ± 0.04 g and hypoxic group with 0.88 ± 0.05 g, $P < 0.01$). Hypoxic embryos hatched at the same time but with smaller masses than normoxic embryos (Table 4-1). In addition, the dry mass of ventricle was similar, but relative ventricular mass and hematocrit was higher, for the hatchlings in hypoxic conditions (Table 4-1).

The critical oxygen tension and oxygen consumption of embryos at day 21 were not different between the control and hypoxic group ($P > 0.05$, Table 4-2). At day 33, embryos that were exposed to normoxic conditions increased their critical oxygen tensions by 9 %, whereas embryos that were exposed to hypoxic conditions reduced their critical oxygen tensions by 24 %. The difference in the critical oxygen tensions of embryos between the two groups was different ($P < 0.001$). Measurements of blood vessels in CAM, including the number of intersections, surface area, and length per unit area, were statistically the same at day 21 for both groups (Table 4-3). By day 33, only the blood vessel length was different ($P < 0.01$) (Table 4-3).

Discussion

Oxygen consumption and growth

The metabolic pattern of normoxic embryos resembles patterns previously reported for Chrysemys and Kinosternon (Lynn and von Brand 1945) in that $\dot{V}_{O_2}$ increases during incubation, then reaches a plateau. The metabolism of normoxic embryos reached a plateau at day 33, suggesting that embryonic growth has reached a maximum level. In contrast, hypoxic embryos

did not grow at the same rate as normoxic embryos, as indicated by lower metabolic levels (Figs. 4-1, 4-2). As a result, metabolism of hypoxic embryos did not reach the plateau at day 33 as normoxic embryos did; consequently, they continued to consume oxygen at an increasing rate and took six more days to reach the maximum level.

Embryos incubated in hypoxic conditions were smaller at day 39 than those in normoxic conditions, indicating that hypoxia retards embryonic growth (Fig. 4-2). Similar findings have been reported for eggs of the green sea turtle, the loggerhead, and the snapping turtle (Ackerman 1981a; Kam 1988). In avian eggs, depression of metabolism and growth by hypoxia has been interpreted as either an inability of embryos to take up enough oxygen from surroundings (Carey et al. 1982; Snyder et al. 1982; Carey et al. 1989), or an adaptive adjustment to maintain air-cell P_{O2} adequate for tissue oxygenation (Wangensteen et al. 1974; Beattie and Smith 1975).

Much information is available on the effects of hypoxia on growth and development of avian embryos. Chicken embryos incubated at moderate hypoxia (at 1500 m, P_{O2} = 125 mmHg) maintain normal oxygen consumption and embryonic growth, and hatch with the same mass as embryos in normoxic condtions (Snyder and Black 1980). Because hypoxic embryos probably have lower air-cell P_{O2}, and, consequently, lower arterial P_{O2}, adjustments must have compensated for the hypoxia. Exposure to severe hypoxia (at 3800 m, P_{O2} = 93 mmHg) results in depressed metabolic rate and growth, prolonged incubation period, reduced hatchling mass, and reduced hatchability (Smith et al. 1969; Wangensteen et al. 1974).

In bar-headed geese (Anser indicus) and Canada geese (Branta canadensis), oxygen consumption, incubation period, and hatchling mass of embryos show no differences between moderate and severe hypoxic groups

(P_{O2} = 125 and 94 mmHg respectively) (Snyder et al. 1982). Similar results are found in the incubation period and hatchability of eggs among populations of horned larks (Eremophila alpestris) breeding at 2400 m and 3800 m (Verbeek 1967; Conry 1978). A study on red-winged blackbirds (Agelaius phoeniceus) over 2900 m altitudinal gradient found no differences in embryonic metabolism, incubation period, and hatchling mass (Carey et al. 1982). In contrast, eggs of montane coots (Fulica americana) laid at 4150 m have depressed metabolic rate and prolonged incubation period, but hatch with the same mass as that of lowland coots (Carey et al. 1989).

Incubation period, hatching success, and hatchling mass.

Exposure to chronic hypoxia resulted in retarded growth, depressed metabolism, comparable incubation period and hatchability, and reduced hatchling mass in red-bellied turtle embryos. Different patterns have been observed in the green and loggerhead turtles in that embryos exposed to more hypoxic and hypercapnic environments grow slowly and hatch later with higher mortality than embryos exposed to less hypoxic and hypercapnic environments (Ackerman 1981a). Furthermore, the size of hatchlings varies among populations. Eggs from Costa Rica hatch with the same size regardless of the treatments, whereas eggs from Ascension Island hatch smaller in the more hypoxic treatments. Another study on loggerhead turtle eggs has shown that incubation period increases but hatchability and hatchling sizes decrease as moisture levels of the incubating media increase (McGehee 1990). Snapping turtle embryos incubated in wet media grow more slowly and hatch later with similar mass and mortality when compared to embryos that are incubated in dry media (Kam 1988).

Embryos incubated in hypoxic conditions could have at least four different outcomes in reptilian and avian embryos. First, embryonic growth is not affected by hypoxia, and embryos have comparable incubation period and hatchling mass as embryos in normoxic conditions. This pattern has been reported in chicken eggs at 1500 m (P_{O2} = 133 mmHg) (Black and Synder 1980); horned lark eggs at 2400 and 3800 m (P_{O2} = 117 and 93 mmHg respectively) (Drury 1961; Verbeek 1967; Conry 1978); red-winged blackbird eggs at 1600, 2400, 2900 m (P_{O2} = 131, 117, 109 mmHg respectively) (Carey et al. 1982). Second, embryonic growth is retarded by hypoxia, and embryos take longer time to grow but hatch with a mass similar to eggs in normoxia. This pattern has been reported in montane coot eggs at 4150 m (P_{O2} = 87 mmHg); green and loggerhead turtle eggs from Costa Rico (oxygen tensions were not reported) (Ackerman 1981a) and snapping turtle eggs (oxygen tensions were not measured) (Kam 1988). Third is a pattern similar to the previous one except that embryos hatch with smaller sizes and mortality is increased. This has been reported in chicken eggs at 3800 m (P_{O2} = 93 mmHg) (Smith et al. 1969; Wangensteen et al. 1974); green turtle eggs from Ascension Island (oxygen tensions were not reported) (Ackerman 1981a) and loggerhead turtle eggs (oxygen tensions were not measured) (McGehee 1990). Fourth, embryonic growth is retarded by hypoxia, and embryos have incubation period and hatchability that are similar to normoxic-raised embryos, but hatchling mass is smaller (P_{O2} = 72 mmHg) (present study).

It is clear that when embryonic growth is retarded by hypoxia, embryos could prolong the incubation period and hatch either with similar mass or smaller mass than normoxic embryos, as found in patterns 2 and 3. In contrast, some embryos do not prolong incubation time and simply hatch smaller, as found in pattern 4. The difference between pattern 2 and 3 can be attributed to

the different severity of hypoxic effects on embryonic growth and development. In pattern 2, hypoxic exposure is not severe, and the physiological functions are limited but not affected. Embryos simply take longer time to grow and hatch later with the same hatchling mass, as found in pattern 2. In contrast, embryos in pattern 3 are exposed to severe hypoxia, and the physiological functions are not only limited but also affected; therefore, embryos would not only grow more slowly but also hatch later with smaller mass and relatively high mortality.

Florida red-bellied turtle embryos in hypoxic conditions hatched at the same time but with smaller mass when compared to normoxic embryos, suggesting that this is a hypoxia-induced hatching event. Previous studies have shown that hypoxia or anoxia induces hatching in the fish, Fundulus heteroclitus, the frog, Pseudophryne bibroni, and the turtle, Carettochelys insculpta (DiMichele and Taylor 1980; Webb et al. 1986; Bradford and Seymour 1988). Hypoxic-induced hatching can only succeed when a size is attained sufficient to break the eggshell and there is a sufficient level of maturity of organ systems. In this study, embryos were subjected to hypoxia only during the second half of the incubation period, which coincides with the end of the differentiation phase and beginning of the growing phase. Thus, embryos were subjected to hypoxia after differentiation had been completed, and hypoxia presumably affects embryonic growth but not differentiation. Similar findings have been reported for chicken embryos in that hypoxia has a greater effect upon differentiation than upon growth at middle stages (Smith et al. 1969).

The mechanism by which hypoxia could initiate hatching in reptilian eggs is poorly understood. In bird eggs, glucocorticoid has been reported to play a major role in hatching of turkey embryos, as indicated by an increase of hatchability after exogenous administrations of glucocorticoid (corticosterone) (Wentworth and Hussein 1985). Glucocorticoid is also important in synthesis of

surfactant in the embryonic lung of chickens right before hatching (Hylka and Doneen 1983). However, it is not known if the glucocorticoid plays the same role in inducing early hatching during hypoxia.

The described patterns may not be the only ways hypoxia influences embryonic growth and development. More patterns will probably emerge as additional studies are done. These patterns may also be confounded by some differences in the experimental protocols. For example, snapping turtle, green turtle and loggerhead eggs are subjected not only to hypoxia but also hypercapnia, and gas concentrations were not constant during incubation (Ackerman 1981a; Kam 1988). Bird eggs are usually exposed to hypoxia at lower barometric pressures, but reptilian eggs are usually exposed to hypoxia at normal barometric pressures. Timing of hypoxic treatment may result in different results. In this study hypoxia was introduced at about 50 % of the incubation period, which is different from previous studies.

Embryonic adjustments to hypoxia

Embryos exposed to chronic hypoxia reduced critical oxygen tension by 24 %, indicating that embryos can increase oxygen transport in response to hypoxia. The increase of oxygen transport is probably the result of adjustments of circulatory (i.e., relative ventricular mass, hematocrit) and respiratory (CAM morphometry) systems. However, embryonic growth in the hypoxic group was lower than in the control group, suggesting that the increase of oxygen transport could only partially compensate for the hypoxic effects. Snyder et al. (1982) incubated eggs of bar-headed and Canada goose at a moderate (P_{O2} = 125 mmHg) and severe (P_{O2} = 95 mmHg) hypoxic conditions. They found that the critical oxygen tensions are lower in the former species. In addition, the critical oxygen tension of bar-headed geese is not affected by severe hypoxia, but is

reduced in Canada geese. Eggs of the bar-headed goose, laid at high altitude (as high as 5500 m), possess adaptations to maintain normal oxygen consumption in moderate and severe hypoxia. In contrast, eggs of the Canada goose, laid at near sea level, do not tolerate hypoxia well; however, they can increase the oxygen transport in response to a severe hypoxic treatment.

Adjustments in response to hypoxia have been reported for avian embryos at the respiratory, circulatory, and tissue levels. Chronic hypoxia stimulates angiogenesis in the CAM of chicken eggs (Dusseau and Hutchins 1988; Strick et al. 1991). Exposure to hypoxia increases hemoglobin-oxygen affinity in chicken and other domestic fowl embryos (Black and Snyder 1980; Baumann et al. 1983) and heart mass of domestic chicken and Canada goose hatchlings (Black and Snyder 1980; Snyder et al. 1982). Hypoxia probably induces a redistribution of blood flow to embryonic tissue such that blood is preferentially distributed to areas with a high priority (e.g. heart) than to areas with a low priority (e.g. limb muscle). Chicks hatched in hypoxic conditions have significantly heavier heart and lighter gastrocnemius muscle when compared to chicks that hatched in the normoxic conditions, even though body masses are the same (Black and Snyder 1980)

The other possible adaptive adjustment of embryos is to modify the resistance of the eggshell-membranes to oxygen diffusion. Eggs laid at high elevations develop in hypoxic conditions and encounter greater water losses due to the increase of the diffusion coefficient of gases at lower barometric pressures. Increased O_2 conductance of the eggshell promotes O_2 uptake but suffers a much greater water loss. The opposite is also true. Some eggs are built to maintain water balance at the expense of maximizing oxygen uptake (Wangensteen et al. 1974, Carey et al. 1982). Consequently, the oxygen requirement for normal development is met by modifying the resistance of the

inner barrier to oxygen diffusion in red-winged blackbirds (Carey et al. 1982). However, coot eggs (Fulica americana) laid at 4150 m have increased O_2 and CO_2 conductance of eggshells, which favor gasous diffusion and increased water loss (Carey et al. 1989).

At lower elevations, hypoxia occurs only when eggs are buried underground, such as in the case of the Brush Turkey (Alectura lathami) and Mallee Fowl (Leipoa ocellata) (Seymour et al. 1986). Here, the selective pressure for eggshell conductance to maintain water balance is relaxed because of the high humidity inside the nest. Thus, a high gaseous conductance of the eggshell is adaptive to facilitate exchange of respiratory gases (Seymour 1985; Seymour et al. 1986).

In conclusion, low critical oxygen tensions at day 21 and day 33 indicate that Florida red-bellied turtle embryos tolerate hypoxia better than bird embryos (Visschedijk et al. 1980; Synder et al. 1982; Seymour 1985). This is probably attributed to the high O_2 conductance of reptilian eggs (Ackerman and Prange 1972; Lutz et al. 1980; Feder et al. 1982) and the low metabolic rate of embryos over long incubation periods (Ackerman 1980). However, embryonic growth was retarded when Florida red-bellied turtle eggs were incubated in hypoxic conditions at P_{O2} = 72 mmHg, indicating that embryos are no longer able to maintain normal metabolism at this hypoxic level. Furthermore, hypoxic embryos grew slower but hatched at the same time as normoxic embryos, suggesting that hypoxia only influences embryonic growth but not development during the latter half of incubation.

The decrease of critical oxygen tensions by 24 % in response to hypoxia indicates that Florida red-bellied turtle embryos are flexible, structurally and physiologically, in enhancing oxygen transport to compensate for hypoxia. These findings are in agreement with previous studies in chicken and Canada

goose embryos (Dusseau and Hutchins 1988; Strick et al. 1991; Baumann et al. 1983; Snyder et al. 1982).

Table 4-1. Hatchling, residual yolk, and ventricular mass, incubation period, and hematocrit of Florida red-bellied turtle hatchlings in normoxic and hypoxic conditions. Values are reported as means with standard error in parenthesis. P is significant probability.

	Normoxic	Hypoxic	P
Sample size	20	23	
Incubation time (days)	43 (1.2)	42 (1.6)	> .05
Wet hatchling mass (g)	6.899 (0.257)	6.184 (0.225)	< .05
Dry hatchling mass (g)	1.608 (0.083)	1.323 (0.059)	< .01
Wet yolk mass (g)	1.352 (0.095)	1.872 (0.070)	< .001
Dry yolk mass (g)	0.696 (0.051)	0.9871 (0.045)	< .001
Wet ventricular mass (mg)	15.2 (0.73)	15.1 (0.94)	> .05
Dry ventricular mass (mg)	2.8 (0.16)	3.2 (0.25)	> .05
Relative ventricular mass (%)	0.18 (0.11)	0.25 (0.19)	<.01
Hematocrit (%)	27 (0.9)	34 (1.3)	< .001

Table 4-2. Oxygen consumption ($\dot{V}_{O_2}$) and critical oxygen tension (P_c) of embryos at day 21 and 33. Values are reported as means with standard error in parenthesis. Sample size is 6 for each group. P is significant probability.

	Normoxic	Hypoxic	P
Day 21			
$\dot{V}_{O2}$ (ml / h)	0.588 (0.041)	0.626 (0.032)	> .05
P_c (mmHg)	94.4 (3.8)	94.5 (3.8)	> .05
Day 33			
$\dot{V}_{O2}$ (ml / h)	1.393 (0.032)	1.271 (0.062)	> .05
P_c (mmHg)	103.7 (4.0)	72.4 (2.8)	< .001

Table 4-3. Morphometry measurements of blood vessels in the chorioallantoic membrane of embryos incubated in normoxic and hypoxic conditions. Values are reported as means with standard error in the parenthesis. Sample size is 6 for each group. P is significant probability.

	Normoxic	Hypoxic	P
No. of intersection			
Day 21	40.4 (2.3)	41.5 (2.9)	> .05
Day 33	45.8 (3.6)	54.2 (3.2)	> .05
Surface area (mm^2)			
Day 21	0.664 (0.020)	0.674 (0.026)	> .05
Day 33	0.895 (0.053)	0.980 (0.076)	> .05
Length (mm)			
Day 21	8.41 (0.58)	8.65 (0.23)	> .05
Day 33	9.17 (0.32)	11.29 (0.42)	< .01

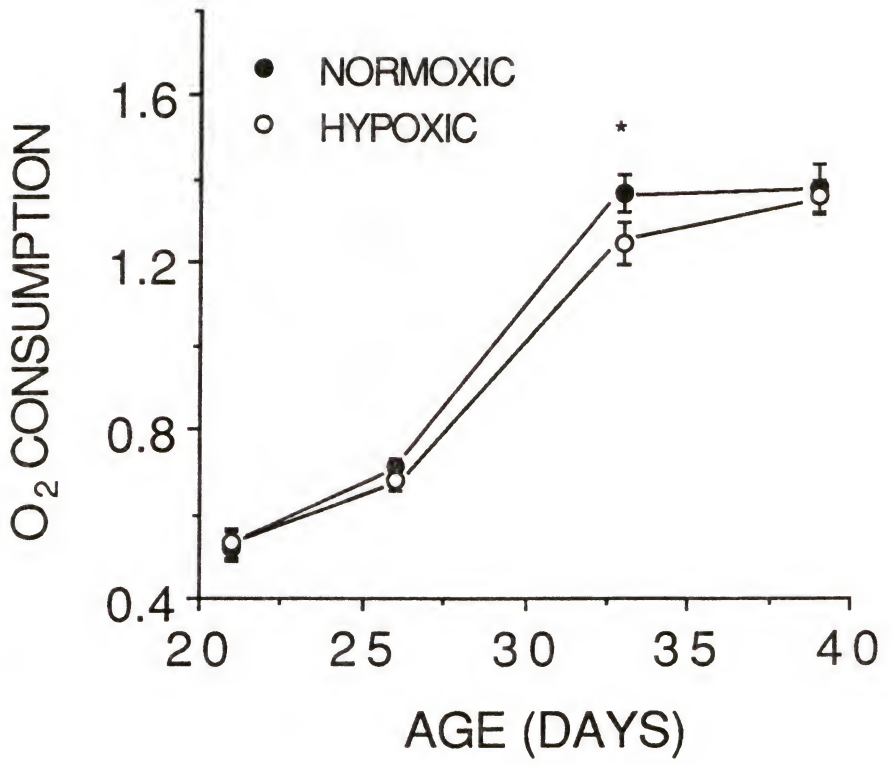

Figure 4-1. Oxygen consumption (ml / h) of embryos incubated in normoxic and hypoxic conditions as a function of incubation days. Values are reported as means ± SE. * represents a significant difference at a 0.05 level.

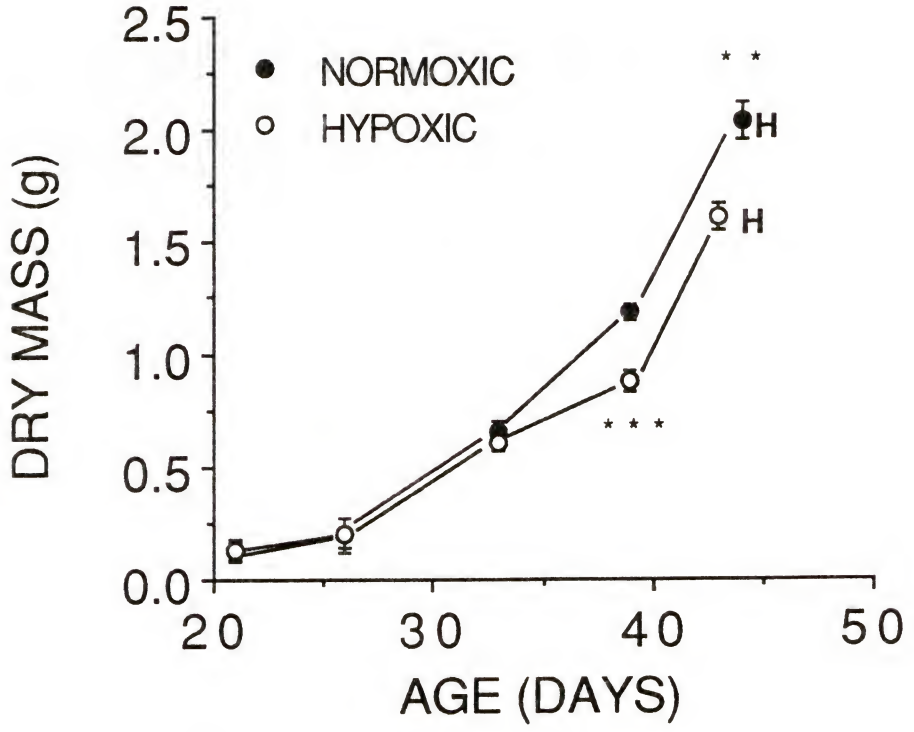

Figure 4-2. The increase of embryonic mass with incubation time. Values are reported as mean + SE. ** and *** represent significant differences at 0.01 and 0.001 levels, respectively. "H" symbols indicate hatchlings.

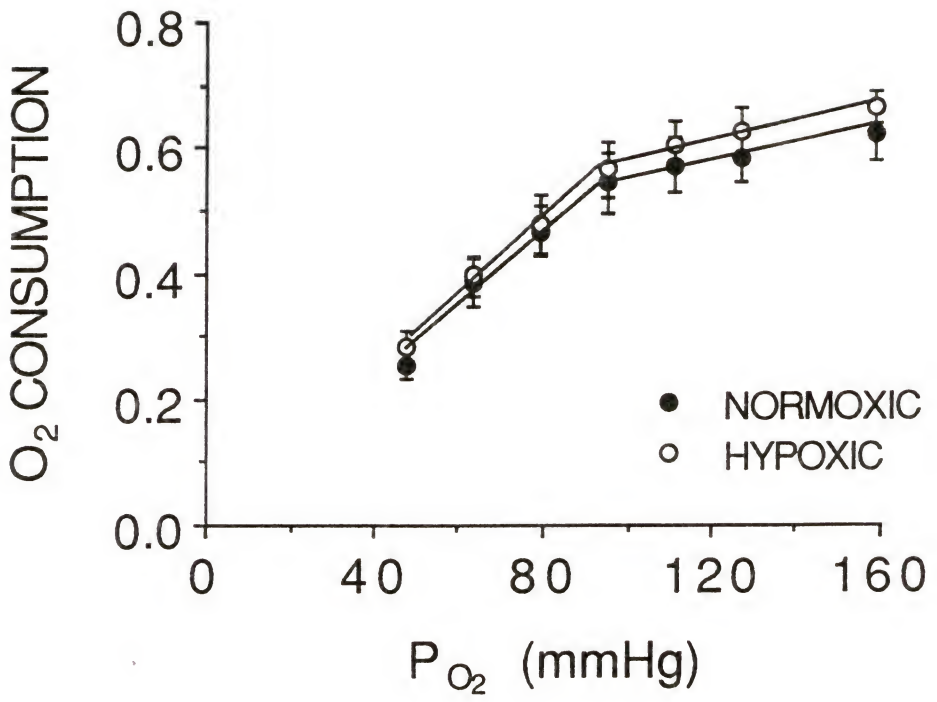

Figure 4-3. Oxygen consumption (ml / h) of embryos at day 21 as a function of oxygen tension. Values are means ± SE.

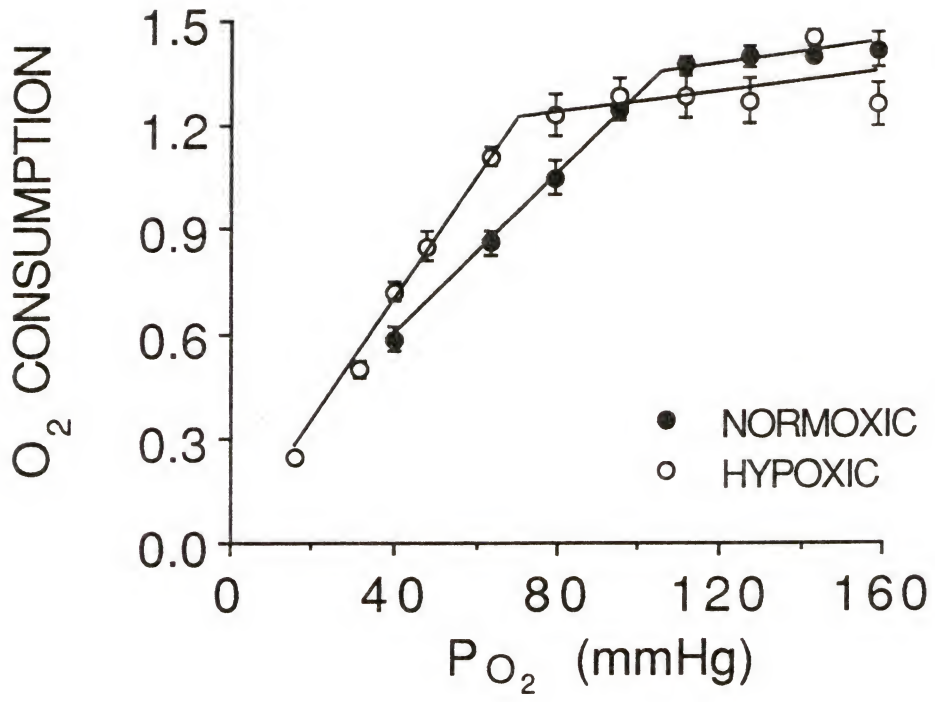

Figure 4-4. Oxygen consumption (ml / h) of embryos at day 33 as a function of oxygen tension. Values are means ± SE.

CHAPTER 5
EFFECTS OF SIMULATED FLOODING ON METABOLISM, WATER BALANCE, AND HATCHING SUCCESS OF FLORIDA RED-BELLIED TURTLE (PSEUDEMYS NELSONI) EMBRYOS

Introduction

Reptilian eggs incubated underground are potentially subjected to flooding when water levels are raised by rainfall, high tides, or other natural phenomena. Laboratory or field studies have demonstrated flooding is a major cause of egg mortality in Crocodylus porosus (Webb et al. 1977), Alligator mississipiensis (Hines et al. 1968, Joanen 1969, Kushlan and Kushlan 1980), and Trionyx m. muticus (Plummer 1976). Egg mortality is dependent on the duration of submergence.

Although studies have been directed at the effect of flooding on egg mortality, no attempt has been made to investigate its influence on water balance and embryonic metabolism and development. When eggs are inundated, the oxygen availability drops, and the eggshells are saturated with water, which favors water exchange but not gas exchange. It is not known how embryos respond to a sudden change from an aerial environment to an aqueous environment. It is unclear if flooding has any effect on embryonic development and metabolism in those embryos that survive.

Many Florida red-bellied turtles (Pseudemys nelsoni) found in Florida wetlands lay their eggs in nest mounds of the American alligator. Eggs are usually located either level with or below alligator nest cavities, which are about 25 cm above the water level (Goodwin and Marion 1977). The nest mounds

have horizontal bases of about 150-160 cm in diameter and 50 cm in height, and in close proximity to permanent water (Goodwin and Marion 1978). An annual survey found that about 20-30 % of nests are flooded during at least some part of the incubation period (G. Masson, personal communication). The purpose of this study was to assess the physiological effects of flooding on water balance, metabolic rate, growth, survivorship, and hatchlings of parchment-shelled eggs and embryos of P. nelsoni.

Materials and Methods

A total of seven clutches of red-bellied turtle eggs were collected from alligator nests in Alachua county, Florida during June and July, 1991. One egg from each clutch was opened to estimate age. Eggs were randomly and equally assigned to a control and three flooding treatments: 1, 3, and 6 days of submersion. Each group of 27 eggs were half-buried in sand with 4 % gravimetric water content and incubated at a constant 30°C. The boxes were covered with plastic sheets to reduce evaporative water loss, and water was added as necessary.

Egg mass and oxygen consumption of 9 randomly selected eggs from each group were measured periodically throughout the incubation starting at day 19. For the control and 1-day submergence treatment, measurements were also made at 1, 3, 6, 9, 16, and 24 days after submersion. For the 3- and 6-day submergence treatments, measurements were made at 3 and 6 days, respectively, after submersion.

Eggs of the submergence groups were incubated in sand throughout incubation except during the flooding experiments. The flooding experiments

were carried out in the same walk-in environmental chamber where eggs were incubated. Eggs were submerged in distilled water (30°C) at 19 days of incubation and removed from water after 1, 3, and 6 days of submersion. Each egg was weighed immediately after removal from water, and oxygen consumption was determined. Because the relative humidity in the metabolic chambers was saturated and the duration for measuring oxygen consumption was short (no more than 45 minutes), it was assumed that the oxygen consumption was measured when the eggshell was still near, if not fully saturated.

The oxygen consumption of eggs was measured using a closed-system, and Glass jars (Kerr Glass Inc.) fitted with two rubber stoppers and three-way stopcocks were used as metabolic chambers. These chambers contained 5 ml of distilled water and a piece of wet filter paper to keep the chambers at 100 % relative humidity. Each chamber was flushed with humid air before stopcocks were closed. The interval for gas sampling was dependent on the developmental stage of embryos such that no more than 1-2 % of total oxygen was consumed. A 40 ml of gas sample was taken at the end of incubation, and the fractional O_2 concentration was determined with an Applied Electrochemistry S-3A O_2 analyzer. Oxygen consumption, $\dot{V}_{O_2}$ (ml STPD / h), was calculated using the equation, $V(FI_{O_2}-FE_{O_2})/(1-FE_{O_2})t$, where V is the dry, CO_2-free air, FI_{O_2} and FE_{O_2} are the initial and final oxygen fraction (%) of a measurement, and t is the duration (h) from start to end of a measurement (Vleck 1987).

Wire partitions were installed in every box when pipped eggs were found in any box. Boxes were checked everyday, and hatchlings were killed by freezing. Later, hatchlings were thawed at room temperature, and wet mass

was weighed. Residual yolk and carcass were separated, weighed, dried at 65 ° C, and reweighed.

Egg mass, hatchling mass, and oxygen consumption were adjusted using analysis of covariance (ANCOVA) and the initial mass was used as covariate. All means were expressed as mean (± standard error) unless otherwise mentioned. Dry hatchling and residual yolk mass were tested for significant difference using ANOVA.

Results

Embryonic metabolism

The oxygen consumption of control eggs increased throughout incubation and reached 1.63 ml O_2 / h at day 43 (Fig. 5-1). All submerged eggs reduced their oxygen consumption to low levels at around 0.07-0.09 ml O_2 / h. Eggs of the1-day submergence treatment resumed oxygen consumption quickly and almost reached the same level as eggs in the control group at 24 days after the flooding experiment was started (i.e., day 43) (1.56 ml O_2 / h, $P > 0.05$) (Fig. 5-1). In contrast, the oxygen consumption of eggs of the 3- and 6-day submergence treatments decreased and did not recover to the levels at day 19 (Fig. 5-2).

Hatching success, incubation period, and hatchlings

The hatching success and incubation time of red-bellied turtle eggs were significantly affected by the duration of submergence (Table 5-1). The percent hatching was highest for eggs in the control group and progressively lowered for eggs in the 1- and 3-day submergence treatments, while no eggs in the 6-

day submergence treatment survived. Eggs in the control required significantly less time to hatch than eggs in the 1- and 3-day submergence treatments ($P < 0.001$, $P < 0.01$ respectively).

The dry masses of carcass and residual yolk and water content of hatchlings were not different among groups (Table 5-2). In the 1-day submergence treatment, the dry mass of hatchlings (1.788± 0.07 g, n=7) was significantly heavier than that of dead embryos (1.384 ± 0.07 g, n=14) ($P < 0.01$). The dry mass of dead embryos of the 1-day submergence treatment was heavier than that of the 3- and 6-day submergence treatments (0.04 ± 0.04 g, n = 20 and 0.05 ± 0.04 g, n=27, $P < 0.001$, $P < 0.001$ respectively), but the latter two were not significantly different from each other ($P > 0.05$).

Egg mass during incubation

Eggs in the control and 1-day submergence treatment increased mass throughout incubation, and the patterns of water uptake were similar (Fig. 5-3). They gained 1.43 and 1.83 g of water, respectively, at 24 days after the flooding experiment started, and the difference was not significantly different ($P > 0.05$). In contrast, eggs in the 3- and 6-day submergence treatments lost water throughout incubation except during submergence (Fig. 5-4). They lost 0.59 and 0.79 g of water respectively at 24 days after the flooding experiments were started, and the difference was not significantly different ($P > 0.05$).

Simulated flooding had significant effects on the water uptake of eggs (Table 5-3). Eggs in the 1- and 3-day submergence treatments gained 84 and 42 % respectively ($P < 0.001$ and $P < 0.05$) more water than eggs in the control within the same duration. However, eggs in the control gained 322 % more water than eggs in the 6-day submergence treatment within the same duration ($P < 0.01$).

Discussion

Patterns of metabolism

The metabolic pattern of control eggs resembles patterns reported for Chrysemys and Kinosternon (Lynn and von Brand 1945) in that $\dot{V}_{O_2}$ increases during incubation, then reaches a plateau. A similar metabolic pattern is exhibited by embryos of precocial birds (Vleck et al. 1980). The $\dot{V}_{O_2}$ of red-bellied turtle embryos reached a plateau at day 35, which was about 78 % of the incubation period (Incubation period = 45 days).

In contrast, the $\dot{V}_{O_2}$ pattern of embryos of the 1-day submergence treatment differed from that of the control group in that $\dot{V}_{O_2}$ increased during incubation but did not reach a plateau (Fig. 5-1). This difference is attributed to the effect of flooding. The embryonic metabolism of the control group reached a plateau at day 35, suggesting that embryonic growth had reached a maximum level. In contrast, the embryonic metabolism of the 1-day submergence treatment was suppressed during submersion and resumed after eggs removal from water. However, $\dot{V}_{O_2}$ was still somewhat lower than the control group, suggesting that embryos grow at slower rates. Embryonic metabolism did not reach the plateau at day 35 as in the control; consequently, embryos continued to grow at an increasing rate and took 8 more days to reach the maximum level.

Embryos in the 3- and 6-day submergence treatments had $\dot{V}_{O_2}$ patterns which differed from those of controls. Metabolism was suppressed during submersion and did not return to the initial values after eggs were removed from water (Fig. 5-2). The low levels of metabolism suggest that physiological processes are permanently damaged during submersion. This hypothesis is

supported by the findings that only one embryo out of 54 eggs survived submersion, and the dry mass of carcasses was not different from that of embryos measured at the day of experiment.

Hatching success

Hatching success was lowest for eggs that were submerged in water for 6 days, indicating that a long duration of submergence is harmful to the developing embryos. Earlier laboratory studies have shown that duration of submergence reduces the hatching success of eggs of Trionyx m. muticus (Plummer 1976), Alligator mississippiensis (Joanen et al. 1977), and Crocodylus porosus (Magnusson 1982). The interaction between duration of submergence and age is important in determining hatching success. In A. mississipiensis, a long duration of inundation (48 h) kills embryos at all ages. Short durations of inundation (2-6 h) yields a high hatching success of eggs at all ages. However, an intermediate duration of inundation (12 h) kills more old embryos than young embryos (Joanen et al. 1977). It appears that in A. mississippiensis, embryos at all ages could tolerate severe hypoxia for at least 6 h, but no more than 48 h. Between 6-48 h, the degree of tolerance is dependent upon the age of embryos, which, in turn, depends largely on the metabolic levels of embryos. Young embryos have comparatively low metabolic rates; therefore, they could tolerate hypoxia longer than older embryos. Kraemer and Bell (1980) reported that loggerhead (Caretta caretta) embryos at all developmental stages are equally vulnerable to the flooding rain, suggesting that the duration of inundation must have well passed the upper limit of the tolerance of embryos.

Whether hatching success is affected by the oxygen content in water is debatable. Theoretically, when eggs are submerged, the air-filled pathways in

the eggshell fill with water, and continuous liquid columns are formed. Consequently, eggs could obtain oxygen via water-filled pathways by diffusion. However, Kutchai and Steen (1971) demonstrated that the O_2 permeability of the compound membranes in a chicken egg is about 30 times higher than that of a layer of water of the same thickness. If this is true for reptilian eggs, then the limiting factor would be the O_2 permeability of the eggshell and not the amount of oxygen in the water.

The duration of anoxia tolerance may be species-specific, but more measurements from other species and a standard experimental protocol are needed before such a generalization can be reached. For example, eggs do not survive in water for 2 days in A. mississippiensis (Joanen et al. 1977), 13 h in C. porosus (Magnusson 1982), 15 days in T. m. muticus (Plummer 1976), and 6 days in P. nelsoni (present study). These differences can largely be explained by the difference in oxygen consumption. The longer duration of tolerance in water for turtle eggs when compared to crocodilian eggs is attributed to the difference in egg sizes and metabolic rates. Furthermore, the difference in tolerance between T. m. muticus and P. nelsoni is because the former was subjected to submersion at a younger age than was the latter (day 12 and 19 with incubation periods of 60 and 44 days at 30 ° C respectively).

Causes of mortality

Flooding kills embryos in two different ways. First, it kills embryos during submergence by limiting oxygen availability, i.e., suffocation. In the 3- and 6-day submergence treatments, metabolism was suppressed during submersion and did not return to the initial values after eggs were taken out of water. All except one embryo in the 3- and 6-day submergence treatments were dead and

dry carcass mass was not different from that of embryos measured at the day of the experiment. Both measurements indicate that physiological processes are permanently damaged during submersion. A heavy rainfall kills embryos of C. caretta by suffocation (Kraemer and Bell 1980). This is inferred because the developmental stages of dead embryos correspond with their ages at the time of being flooded by rainfall.

Second, flooding kills embryos during the late incubation period by some unknown mechanism(s). Based on patterns of $\dot{V}_{O_2}$, embryonic growth in the 1-day submergence treatment was not affected by flooding except during submersion. Submersion experiments were started when embryos were at 19 days of incubation, and the $\dot{V}_{O_2}$ of embryos until 24 days later were periodically measured . The normal $\dot{V}_{O_2}$ pattern suggests that embryos were alive at least up to day 43, 4 days before hatching. Because only 25 % of eggs in this treatment hatched, the remaining 75 % of eggs must have died a few days before hatching.

McGehee (1990) incubated C. caretta eggs in sand with 0, 25, 50, 75, and 100 % moisture and found that the hatching success is largest at 25 % moisture and significantly less at higher and lower moisture levels. This suggests the cause of mortality is due to oxygen depletion and possibly carbon dioxide accumulation in nests. Kam (1988) found gas conductance of the incubating media is correlated to moisture levels such that snapping turtle embryos incubated in sand with 12 % gravimetric water content, which have lower gas conductance, grow slower than those with 4 % gravimetric water content.

Incubation period and hatchling size

Incubation time increased as duration of submergence increased. The long incubation period is attributed to suppressed metabolism during submersion (Fig. 5-1). The similarity of dry mass in hatchling and residual yolk of control and treatments suggest flooding does not affect overall embryonic development and growth. Although embryos suppressed metabolism and delayed development and growth during submersion, they attained normal hatching size by taking a longer time to grow.

Water balance

During submersion, the daily water uptake by eggs in the 1- and 3-day submergence treatments was higher than in controls (Table 5-3). When submersed, air-filled pathways are presumably flooded with water. The amount and direction of water exchange between embryos and surroundings depend on the water potential gradient and water conductivity of the eggshell and its membranes. Distilled water has a water potential of zero, whereas the water potential of turtle eggs is about - 700 to - 800 kPa, depending on the osmotic pressure in embryos (Tracey et al. 1978). Thus, water moves from the surroundings to eggs in liquid water. In contrast, eggs of the control were incubated in sand with 4 % gravimetric water content and a water potential of about -4 to -7 kPa (Kam and Ackerman 1990), which does not differ much from that in water. Eggs in the control exchange water mainly via vapor phase, and the water vapor conductivity is much lower than the saturated hydraulic conductivity of eggs in water (Hillel 1982). Consequently, water uptake by eggs in the control group was less than those in 1- and 3-day submergence treatments. It is surprising to find that eggs in the 6-day submergence treatment

gained less water than control eggs even though eggs in the water apparently have higher water conductivity than eggs buried in the sand.

After being removed from water, eggs either continued to increase in mass (control and the 1-day submergence treatment, Figs. 5-1, 5-3), or they started to lose mass (3- and 6-day submergence treatmentsm Figs. 5-2, 5-4). This difference in water balance apparently is related to whether embryos are alive after removed from water. Eggs in the 1-day submergence treatment consumed oxygen at the same rate as eggs in the control, indicating that embryos are still alive. In contrast, eggs in the 3- and 6-day submergence treatments did not resume oxygen consumption at the previous levels, indicating that embryos may have died due to permanent damage to physiological processes of the embryos. It is unclear why the viability of embryos could reverse the direction of the water exchange process, even though the water potential gradient clearly favors water moving from the surroundings into eggs.

Ecological considerations

The hatching success of embryos was related to the duration of submergence, suggesting that red-bellied turtle embryos do not have particular physiological adaptations to flooding. This is probably also true for eggs of A. mississipiensis, C. porosus, and T. m. muticus (Plummer 1976; Joanen et al. 1977; Magnusson 1982). Obviously, flooding is an important factor in determining the nesting success of these species. Trionyx m. muticus experienced 3 % nesting success in a unusually wet year when compared to a 65 % nesting success in a dry year (Plummer 1976). All C. porosus eggs laid beside rivers are killed by flooding (Magnusson 1982) . Numerous field studies have reported that all or nearly all C. caretta eggs are killed by excessive

rainfall (Ragotzkie 1959; Dean and Talbert 1975; Kraemer and Bell 1980), suggesting that C. caretta eggs also do not possess physiological adjustments to flooding.

For those species that bury eggs in habitats where flooding regularly occurs, flooding presumably acts as a strong selective force in the evolution of physiological adaptation of eggs. One possible adaptation available only to well-developed embryos is to hatch when flooding occurs. Treatments such as submergence or perfusion with nitrogen gas induce hatching of Carettochelys insculpta embryos (Webb et al. 1986). Anoxia-induced hatching not only prevents embryos from suffocation, but also supplies more water for hatchlings to disperse.

The other possible adaptation is developmental arrest, which is characterized by low metabolic rates and allows embryos to tolerate adverse conditions (Ewert 1985). The developmental arrest has been documented in eggs of some winter-nesting turtles when ambient temperatures are below optimum. They go through the winter with extremely slow growth and development. Such arrested development ends upon the arrival of warm temperatures, and embryos begin to develop and grow again. As a result, the incubation periods are much longer those of summer breeders (Goff and Goff 1932; Goode and Russell 1968). Embryonic diapause prevents embryos from developing under less optimal conditions, which may lead to gross abnormalities and even death.

There is no evidence at the present time that reptilian embryos exhibit developmental arrests in response to flooding; however, it is possible that developmental arrest evolves as a preadaption (exaptation). For species that exhibit embryonic diapause in response to cold temperatures, the diapause

could be a preadaptation for flooding. In other words, embryos that are in diapause during winter would not be affected by flooding.

For those eggs that are vulnerable to flooding, nest site selection of females becomes important in determining the nesting success and recruitment of young to a population. First, females could nest in different kinds of habitat such that if eggs in a particular habitat are killed by flooding, hatchlings from non-flooded habitats could disperse and replenish the population. Female C. porosus lay eggs not only along the river banks but also in swamp habitats (Magnusson 1982). No swamp nests are flooded, and predation by animals and humans are also lower when compared to nests on river bank habitats. Second, if females choose to nest in a particular habitat, then microhabitats that are least susceptible to flooding are preferred. Caretta caretta nests on high sloping beaches backed by rounded dunes or vegetation, and are usually above the high tide line (Baldwin and Lofton 1959; McGehee 1990). In flatback turtles, Chelonia depressa, 94 % of nests are located on the top of the dunes or on the steep seaward slope, and the remaining 6 % are located between the foot of dunes and the high tide mark (Limpus 1971). Nests of Graptemys oculifera and G. pulchra are clumped at the highest points of open sand bars along the edge of woods (Anderson 1958). The females of T. m. muticus preferably nest on open sandbars free of vegetation, weeds, and sediment markings, but the nest elevation varies among geographical populations. In Kansas, nests are clumped at the highest points of sandbars (Fitch and Plummer 1975: Plummer 1976), whereas, in Iowa, Louisiana, and Mississippi, they are scattered along level sandbars (Goldsmith 1944; Anderson 1958). Goldsmith (1944) reported that during low water level, females nest on the sandy shores between the low and high water marks. These nests are inundated when water level rises.

Table 5-1. Survivorship and incubation time from eggs of the Florida red-bellied turtle subjected to various durations of submersion.

	Control	1 day	3 days	6 days
No. eggs	27	27	27	27
No. live hatchlings	24	7	1	0
% hatched	89	26	4	0
Incubation time (days)	43.5 (0.2)	46.7 (0.8)	47	

Table 5-2. Hatchling and residual yolk mass of Florida red-bellied turtle eggs subjected to various durations of submersion. Values are reported as mean ± SE.

	Control	1 day	3 days	P
Sample size	13	7	1	
Dry hatchling mass (g)	1.87 (0.05)	1.79 (0.07)	1.79	> .05
Dry yolk mass (g)	0.65 (0.02)	0.65 (0.03)	0.59	> .05

Table 5-3. Water uptake by eggs at various durations in sand and water. Values are reported as mean ± SE. Sample size = 9.

	Daily water uptake (g / day)		
	Half-buried in sand	Submerged in water	P
1 day	0.119 (0.005)	0.220 (0.027)	< .001
3 days	0.287 (0.009)	0.407 (0.054)	< .05
6 days	0.520 (0.028)	0.123 (0.144)	< .01

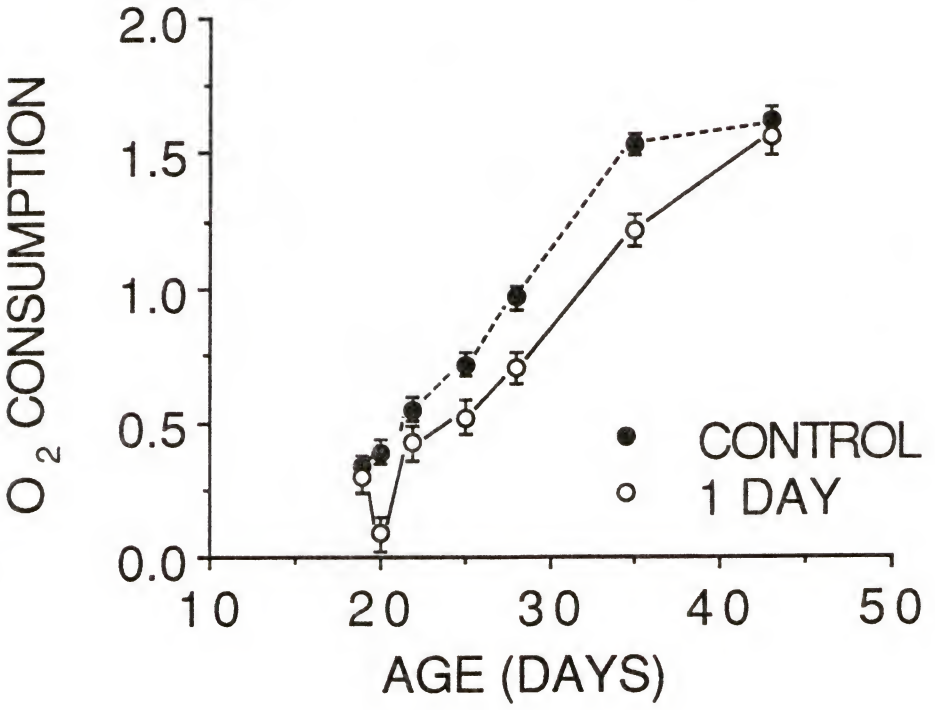

Figure 5-1. Oxygen consumption (ml / h) of eggs of the control (dashed line) and 1-day submergence treatment (solid line) as a function of incubation days. Values are reported as means ± SE.

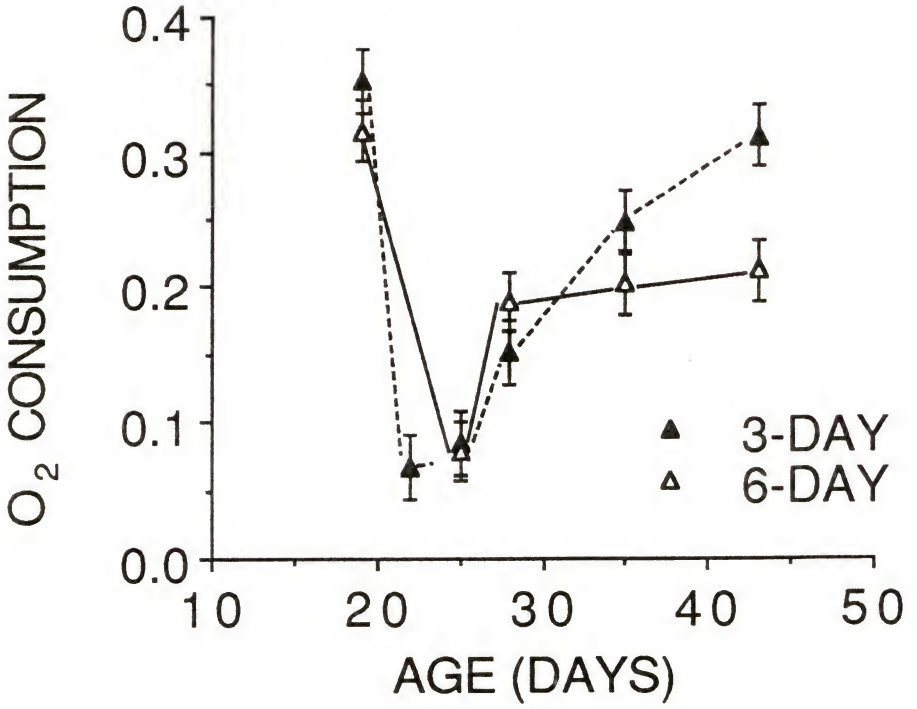

Figure 5-2. Oxygen consumption (ml / h) of eggs of 3- (dashed line) and 6-day submergence (solid line) treatments as a function of incubation days. Values are reported as means ± SE.

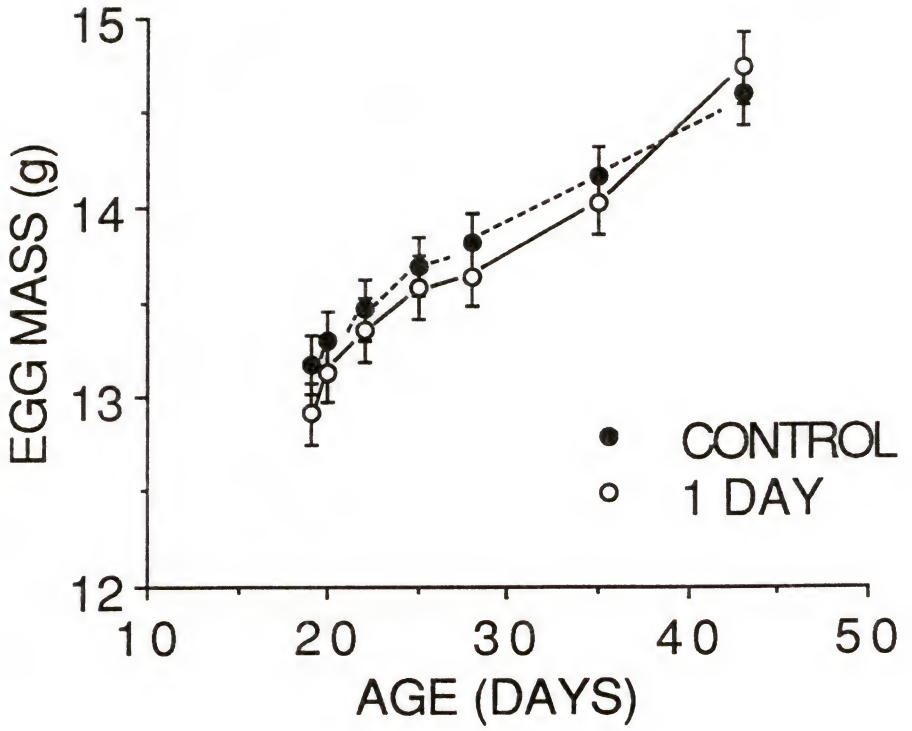

Figure 5-3. Changes in mass of eggs in the control (dashed line) and 1-day submergence treatment (solid line). Values are reported as means ± SE.

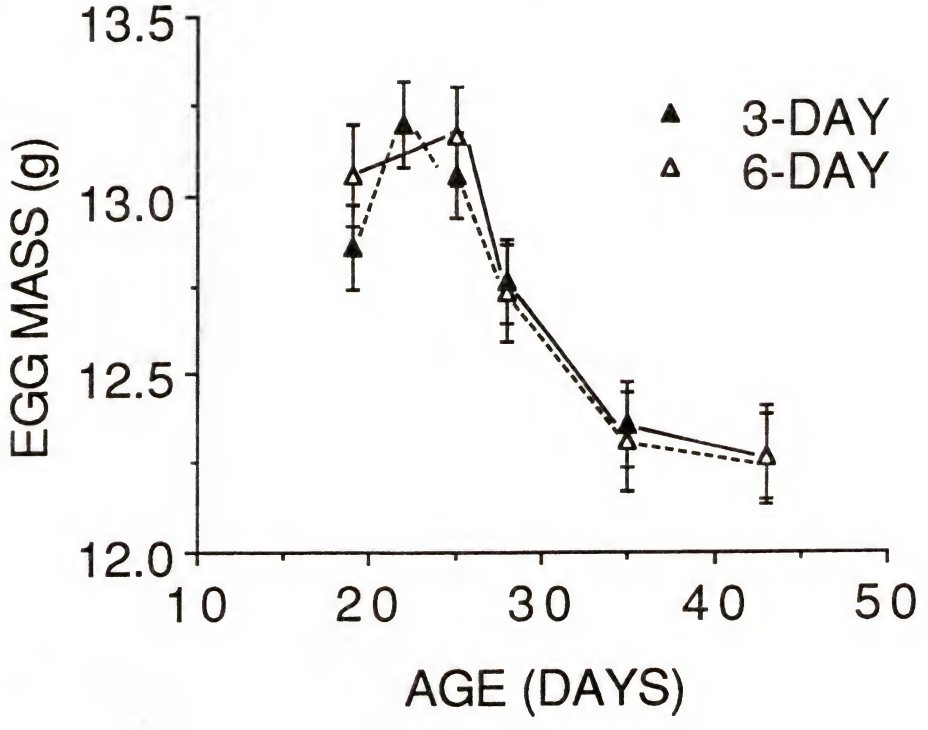

Figure 5-4. Changes in mass of eggs in 3- (dashed line) and 6-day submergence (solid line) treatments. Valus are reported as means ± SE.

GENERAL DISCUSSION

This study focused on gas exchange and physiological responses of reptilian embryos to acute and chronic hypoxia. I determined critical oxygen tensions of turtle, squamate, and alligator embryos in to evaluate their tolerance of hypoxia. I chose the Florida red-bellied turtle, P. nelsoni, as a model organism to further examine environmental influences on gas exchange and physiological responses of embryos to chronic hypoxia and flooding.

Embryos of the American alligator, Alligator mississippiensis, the loggerhead sea turtle, Caretta caretta, the Florida red-bellied turtle, Pseudemys nelsoni, the yellow rat snake, Elaphe obsoleta quadrivittata, and the southern fence lizard, Sceloporus undulatus undulatus maintained a constant $\dot{V}_{O_2}$ to some degree of hypoxia. This indicates that they are metabolic regulators. A low $\dot{V}_{O_2}$ and a high eggshell conductance for O_2 explain how reptilian embryos are able to maintain their metabolism in hypoxic conditions. Hypoxic tolerance decreased over incubation, as indicated by an increase in P_c. Embryonic development characterized by high $\dot{V}_{O_2}$ increases the P_c, whereas the development of circulatory and respiratory functions that promote the G_c reduce the P_c. The P_c increased presumably because increments of G_c, a measure of O_2 transport capacity, did not offset the increase of $\dot{V}_{O_2}$ during development.

The similarity of P_c of embryos between species during late incubation suggests that hypoxic tolerance of reptilian embryos is independent of shell type and egg size. The ability of embryos to tolerate hypoxia does not correlate

with their habitat. For example, C. caretta embryos are the least hypoxic tolerant species even though they clearly experience hypoxia when the P_{O_2} of nest chambers decreases rapidly during the second half of incubation (Ackerman 1977). In contrast, E. o. quadrivittata and S. u. undulatus embryos tolerate hypoxia best, yet these species are shallow nesters and most likely do not encounter hypoxia conditions. Eggs are laid in hollow, rotting logs and vegetation, under railroad ties, beneath rocks, at the base of grass clumps and sawdust piles (Ashton and Ashton 1985; Ashton and Ashton 1988; Ernest and Barbour 1989).

I used Florida red-bellied turtle eggs as model organisms to study effects of temperature and hydration on critical O_2 tension curves. Results show that temperature but not hydration has significant effects on the critical oxygen tension of embryos. Oxygen consumption and P_c of embryos were significantly affected by temperature. The Q_{10} values of 2-3 at different incubation days indicate thermal sensitivity of metabolism remains the same during development. At each incubation day, P_c increased directly proportional to temperature, indicating it is temperature dependent. Oxygen consumption and P_c of the turtle embryos were not affected by egg hydration. Eggs incubated in 13 % gravimetric water content gained mass faster than at 3 % gravimetric water content. However, oxygen consumption, critical oxygen tension, and hatchling mass did not differ between groups.

Critical oxygen tensions at different ages, temperatures, and hydrations were measured when embryos were exposed to acute hypoxia. Generalizations about the ecological adaptation of reptilian embryos to hypoxic conditions in the field may be premature because information concerning the responses of embryos to chronic hypoxia is not available. Thus, the physiological responses of turtle embryos to chronic hypoxia was subsequently

studied. Exposure to chronic hypoxia (10 % air) resulted in retarded growth, depressed metabolism, comparable incubation period and hatchability, and reduced hatchling mass. However, embryos are flexible, structurally and physiologically, in enhancing oxygen transport to compensate hypoxic effects. Hypoxic embryos reduced critical oxygen tensions by 22 mmHg from day 21 to 33, whereas normoxic embryos increased critical oxygen tensions by 9 mmHg. The reduction in the critical oxygen tension of hypoxic embryos indicates that they are capable of increasing oxygen transport in response to hypoxia, which is the result of adjustments of circulatory (i.e., relative ventricular mass, hematocrit) and respiratory (CAM morphometry) systems.

Anoxia is an extreme case of hypoxia, and it could occur when eggs are flooded. About 20-30 % of alligator nests are flooded during at least some part of the incubation periold. Turtle eggs were studied to assess the physiological effects of flooding on water balance, metabolic rate, growth, survivorship, and hatchlings of parchment-shelled eggs and embryos. Results show that simulated flooding has dramatic effects on water balance, embryonic metabolism and development of embryos. The 1-day submergence treatment did not affect water balance and oxygen consumption of embryos, but did increase egg mortality. The 3- and 6- day submergence treatments affected water balance, oxygen consumption, and hatching success of eggs. Flooding kills embryos either during submergence by suffocation, as found in the 3- and 6-day submergence treatments, or during late incubation by some unknown mechanism(s), as found in the 1-day submergence treatment.

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BIOGRAPHICAL SKETCH

Yeong-Choy Kam was born in Negeri Sembilan, Malaysia on October, 7, 1959. He lived in Malaysia, Taiwan, Milwaukee, and Ames before moving to Florida. He graduated from Chung Hwa High School in Kluang, Johore, in 1974. He received a Bachelor of Science degree from the National Taiwan University in 1982. He graduated from the Iowa State University in 1988 with a Master of Science in zoology. He married Chiung-Fen, Iris, Yen on January 1, 1985, and has a son, Michael, and will have a daughter. He is a member of the American Society of Zoologists and the American Society of Ichthyologists and Herpetologists.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Harvey B. Lillywhite,Chair
Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John F. Anderson
Associate Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the Department of Zoology in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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