Water exchange in reptile eggs: mechanism for transportation, driving forces behind movement, and the effects on hatchling size

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Water exchange in reptile eggs:
Mechanism for transportation, driving forces behind movement,
and the effects on hatchling size

by

Todd Alan Rimkus

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Zoology and Genetics
Major: Zoology

Approved:
Signature was redacted for privacy.

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For the Major Department
Signature was redacted for privacy.

For the Graduate College
Iowa State University
Ames, Iowa

1996
DEDICATION

This dissertation is dedicated to Mary Lynne Blaszczyk. Mary Blaszczyk has had and will continue to have the greatest impact on my success. She showed me that by being interested in the work of others you in turn drive them to do their best. As one of only a handful of people who took the time to proof-read and read my masters thesis, I am greatly indebted to her. Because of her interest in me, I was inspired to excel in this field and work toward my doctoral degree. I am saddened that she will not physically see me receive my doctoral degree as she was diagnosed with terminal cancer in 1994 and lost the fight in October of 1995. I know she knows how much she meant to me, because I wrote her a letter in December of 1994 which expressed my feelings about her impact on me as a person and as a scholar. I truly believe that she understands the positive impact that she has on me. I thank her for letting me be touched by her spirit.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Dissertation Organization</td>
<td>3</td>
</tr>
<tr>
<td>Review of Literature</td>
<td>4</td>
</tr>
<tr>
<td>LIQUID AND VAPOR WATER EXCHANGE IN SNAPPING TURTLE (Chelydra serpentina) EGGS THROUGHOUT INCUBATION</td>
<td>9</td>
</tr>
<tr>
<td>Abstract</td>
<td>9</td>
</tr>
<tr>
<td>Introduction</td>
<td>9</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>11</td>
</tr>
<tr>
<td>Source and Condition of Eggs</td>
<td>11</td>
</tr>
<tr>
<td>Incubation Procedure for All Eggs</td>
<td>11</td>
</tr>
<tr>
<td>Experimental Procedure for Fluorescein Experiments</td>
<td>12</td>
</tr>
<tr>
<td>Experimental Procedure for Eggshell Water Content Experiment</td>
<td>13</td>
</tr>
<tr>
<td>Statistical Design</td>
<td>15</td>
</tr>
<tr>
<td>Results</td>
<td>15</td>
</tr>
<tr>
<td>Discussion</td>
<td>18</td>
</tr>
<tr>
<td>Eggshell Drying</td>
<td>18</td>
</tr>
<tr>
<td>Transportation of Water</td>
<td>19</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>22</td>
</tr>
<tr>
<td>Bibliography</td>
<td>22</td>
</tr>
<tr>
<td>EFFECTS OF MAINTAINING VARIOUS CONSTANT WATER POTENTIALS ON HATCHLING SIZE AND WATER EXCHANGE OF EGGS FROM SNAPPING TURTLES (Chelydra serpentina)</td>
<td>35</td>
</tr>
<tr>
<td>Abstract</td>
<td>35</td>
</tr>
<tr>
<td>Introduction</td>
<td>36</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>37</td>
</tr>
<tr>
<td>Source and Condition of Eggs</td>
<td>37</td>
</tr>
<tr>
<td>Experimental Procedure</td>
<td>38</td>
</tr>
<tr>
<td>Statistical Design</td>
<td>40</td>
</tr>
<tr>
<td>Results</td>
<td>42</td>
</tr>
<tr>
<td>Water Exchange</td>
<td>42</td>
</tr>
<tr>
<td>Size Variables</td>
<td>43</td>
</tr>
<tr>
<td>Discussion</td>
<td>45</td>
</tr>
</tbody>
</table>
ABSTRACT

The mechanism for water transport in reptile eggs is examined with particular interest in the phase water is in while being exchanged. Eggs were examined at various times during the incubation. A non-volatile dye was used as an indicator of liquid water movement. Presence of the dye after 48 hours of exposure may be indicative of liquid water movement. If the egg changes mass and no dye is present the water was considered to be exchanged in the vapor phase. Eggs exposed to the dye early during incubation took up the dye, while eggs exposed after day ten have dye levels very close to zero. It is therefore concluded that the majority of incubation is dominated by vapor water transport as the transport mechanism for water in reptile eggs.

The role of water exchange in determining hatchling size was also assessed. Eggs were incubated under constant water potential conditions that would lead to differing amounts of water exchange. Hatchling size was then assessed using length and mass measurements and generating a size index using principle components. The hatchlings from eggs held on the substrate that yielded the highest water uptake were not the largest. The remaining treatments yielded hatchlings that were successively smaller on substrates that had increasingly limited water uptake. In addition to looking at constant water potentials, some eggs were exposed to shifted conditions during three periods of incubation. The hatchlings from eggs encountering successively higher periods of unfavorable conditions were smaller. Eggs exposed to unfavorable conditions during the first period of incubation produced hatchlings that were smaller than hatchlings from eggs
under more favorable conditions. The first period was the most important in determining hatchling size, independent of the conditions encountered in the second or third period, even though the water exchange was most greatly affected by the third period. If a female can select nest sites dependent upon moisture then a female can provide an advantage to her hatchlings by laying in a favorable site even if later during incubation the conditions of the nest become unfavorable.
GENERAL INTRODUCTION

Eggshells in reptiles function to provide an environment that will facilitate embryonic development. The egg contains all of the nutrients necessary for growth. Only water and respiratory gases will be exchanged with the surrounding environment. Therefore, in addition to providing a protective cover, the eggshell must also be porous, in order to allow a pathway for respiratory gases and water to be exchanged with the environment. The necessity of carbon dioxide removal and oxygen uptake is well documented (Lynn and von Brand 1945; Ackerman and Prange 1972; Ackerman 1977; Paganelli et al. 1978; Ackerman 1980), although the necessity of water exchange in reptile eggs is not fully understood. It is known, however, that bird eggs do not develop properly unless they lose 15% of their mass as water (Davis et al. 1988; Lundy 1969). Additionally, reptile eggs that lose in excess of 20% of their mass die during the incubation process (Rimkus unpublished data). The general trend observed in reptile eggs is an overall mass gain during the incubation process (Ratterman and Ackerman 1989; Tracy and Snell 1985; Kam and Ackerman 1990; Tracy et al. 1978; Packard and Packard 1984; Ackerman et al. 1985a; Packard et al. 1979a; Packard et al. 1981).

The environment utilized by the female depositing the eggs will ultimately determine if the egg is under conditions that will drive mass gain or loss. If water is able to move between the egg and the environment, then the water potential difference will determine which way water will move. Water always moves toward the lower or more negative water potential. Therefore, the water potentials of an egg and its environment are
very important if water gain or restriction of loss is necessitated. Few attempts have been made at measuring the water potential of reptile eggs (Muth 1981). If the water potential of the substrate is maintained and can be measured, the driving force could be estimated from the egg mass changes.

The mechanism by which water is able to be transported between the interior of the egg and the environment has not been fully described. Movement of water across the eggshell can be as vapor (Ackerman et al. 1985b), or possibly as a liquid (Thompson 1987). The reason a distinction needs to be made between these two modes of transport, again relates back to the issue of transporting respiratory gases, as well as water. In order to accomplish both water movement and respiratory gas exchange simultaneously, the pores of the eggshell will have to conform to one of the following conditions. The thickness of the shell would have to be sufficiently thin to allow the diffusion of oxygen and carbon dioxide through the liquid filled pores of the eggshell. Alternatively, the water would have to be in a vaporized state to allow for the diffusion of both respiratory gases, as well as, vaporized water. Or thirdly, the pores of the eggshell would have to vary in size so that the smaller pores could transport water, while gases could be exchanged across the larger air filled pores.

An opaque patch on the eggshell has been observed and spreading of this patch has been recorded in some reptile eggs (Thompson 1985). The significance of the patch is likely a drying of the eggshell consistent with the laying down of the chorioallantoic membrane. Therefore, the opaque patch is likely the result of drying down of the eggshell
in order to allow for gas exchange to occur. The changes in these eggshell properties over
the length of incubation should be examined in further detail.

Water uptake independent of the phase it is in while being transported may have an
effect on hatchling size (Gutzke and Packard 1986; Janzen et al. 1990; Packard et al. 1987;
Packard et al. 1988; Packard and Packard 1988). Therefore, if eggs are deposited in
substrates that do not allow sufficient water uptake, this may result in hatchlings that are
smaller. Although the effects of water exchange on hatchling size have been examined by
Gutzke and Packard (1986), as well as, Packard and Packard (1988), the eggs used in their
experiments all lost mass at the end of incubation regardless of the water potential of the
incubation medium. The mass loss observed is likely the result of using vermiculite as an
incubation medium. Vermiculite does not dissipate the metabolic heat produced by the
egg, and the rise in temperature causes a flux of vapor out of the egg consistent with the
observed mass loss (Kam and Ackerman 1990). Therefore, further study of the interplay
between water exchange and hatchling size is warranted. Additionally, size may also
depend on when water is gained or lost.

Dissertation Organization

This dissertation uses an alternate format. The general introduction addresses the
overall problem. The three chapters following the general introduction each address a
specific problem and will be submitted as papers to a scholarly journal for publication.
The dissertation ends with a general conclusion summarizing the research as it pertains to
the overall problem. Following the general conclusions is a list of references cited in the general introduction and the general conclusions.

Review of Literature

Many reptiles excavate subterranean nests to incubate their eggs. The natural nesting environments, ranging from sand to decaying leaf litter, have been well documented (Caldwell 1959; Carr and Ogren 1960; Mortimer 1990; Lutz and Dunbar-Cooper 1984). Female turtles come from the water and lay their eggs in self constructed nests above the water table. The eggs incubate for 50 to 100 days with no additional care. While these eggs are underground, many physiological processes occur. During the incubation process, eggs have been observed to gain, as well as, lose mass (Packard and Packard 1979; Tracy 1980; Andrews and Sexton 1981; Ackerman et al. 1985a; Leshem and Dmi'el 1986). The general pattern seen includes an early period where mass is relatively unchanged with slight losses, gains, or no change in mass reported (Thompson 1987; Rimkus unpublished data; Ratterman and Ackerman 1989; Packard et al. 1984). Then the egg begins to increase in mass and continues to do so throughout incubation. Turtle eggs have been observed to increase in mass from 101% to 140% of original mass (Ratterman and Ackerman 1989; Tracy and Snell 1985; Kam and Ackerman 1990; Tracy et al. 1978; Packard and Packard 1984; Ackerman et al. 1985a). Some have reported mass loss at the end of incubation (Packard et al. 1979a; Packard et al. 1981; Packard and Packard 1984; Tracy and Snell 1985). The phenomenon of late mass loss is most likely associated with
the substrate used as an incubation medium (Kam and Ackerman 1990). The substrate used was vermiculite, a material that has a thermal conductivity 2.8 times less than sand. The eggs incubated in vermiculite will begin to heat at the end of incubation due to metabolic processes. It is this heat that causes the water loss observed (Kam and Ackerman 1990).

The observed mass changes are the direct result of water exchange. The phase that the water is in while being exchanged has not been fully explored. The possibility of the water being in the liquid phase would mean that the pores of the eggshell would be flooded with water during the exchange process. In order for oxygen to be exchanged when all pores are flooded, the oxygen must be dissolved in the water. If water is exchanged in the vapor phase, this would allow for oxygen to be exchanged simultaneously through the non-flooded pores. Another possibility is that the egg exchanges liquid, as well as, vaporized water depending on the developmental state of the embryo. The egg may be able to get the required oxygen during early development from liquid water, but during the later stages of incubation, it is not likely that the oxygen demand can be satisfied through liquid water pathways. There also exists the possibility of liquid and vapor exchange occurring simultaneously. For this to be possible the eggshell would have to be composed of pores varying in diameter (Hirsch 1983; Packard and Packard 1979; and Packard et al. 1982). This arrangement would allow the smaller pores to be saturated while the larger pores might be gas filled. Another problem associated with the movement of any liquid water
would be the possibility of contaminants dissolved in water and smaller than the pores (<10 \mu m in diameter) of the eggshell crossing into the egg.

Thompson (1987) concluded that liquid water is exchanged; however, the experimental results, on which this conclusion was based, were ambiguous. The experiment used freshly laid eggs, half buried in sand with a fluorescent dye present in the sand to monitor liquid water flow. The dye was fluorescein sodium, a non-volatile dye, and therefore a good indicator of liquid water movement. After a 48 hour period, the dye had crossed the shells of the eggs. This indicated that a liquid water pathway existed under the experimental conditions used. The use of freshly laid, still wet eggs ensured that the fully saturated pores of the eggshells were in contact with the substrate. Of greater interest, the eggs in this experiment all lost water. The liquid water path depicted between the eggs and the environment is, therefore, not easily visible. The dye was moving one way and the water was moving in the opposite direction. Additionally, the experiment was only conducted during the first two days of incubation. Therefore, the liquid connection reported may only exist for a short period early in incubation. Liquid water exchange during early incubation may occur, but a liquid connection throughout incubation is unlikely.

Water exchange has been shown to affect size (Packard et al. 1983; Packard et al. 1993). Less negative water potentials produce larger hatchlings with less residual yolk (Packard et al. 1981). The incubation time of eggs in less negative water potentials is extended, which may account for the observed differences in hatchling size and reduced
residual yolk (Packard et al. 1983). The differences observed in a natural setting (Packard et al. 1993) could also have been explained by temperature differences observed. The less negative water potentials were accompanied by cooler temperatures, leading to larger hatchlings with smaller residual yolks. Gutzke and Packard (1986) shifted the water potential of *Chrysemys picta* eggs at the end of each trimester of incubation and found that the first trimester plays little to no role in predicting hatchling size. Therefore, any choice by a female of one particular site over another may be in vain if the hydric conditions change later during incubation. It is also noted that "compensatory water exchange" is occurring in the second trimester but not the third. "Compensatory water exchange" acts to adjust water flow to make up for an excess or a shortage from the previous trimester.

Additionally, Packard and Packard (1988) examined the effects of shifting hydric environments on *Chelydra serpentina* eggs. "Compensatory water exchange" was not observed at any stage of incubation and by far the greatest predictor of size was the third trimester. Weather events were considered to be of greater consequence than nest site selection by the female in determining size of hatchlings. The incubation medium for both of these experiments was vermiculite. The effects of this unnatural substrate may have acted to overshadow effects of the early hydric conditions. The eggs in this experiment all lost mass in the final third of incubation, independent of water potential. The cause for the observed losses was again due to the metabolic heating of eggs in vermiculite (Kam and Ackerman 1990). These late losses did affect the dry mass of hatchlings and residuals yolks (Gutzke and Packard 1986). The use of an unnatural incubation medium has likely
confounded the results; therefore, the effects of water exchange on size requires further examination.
LIQUID AND VAPOR WATER EXCHANGE IN SNAPPING TURTLE (*Chelydra serpentina*) EGGS THROUGHOUT INCUBATION

A paper to be submitted to Physiological Zoology

Todd A. Rinkus and Ralph A. Ackerman

Abstract

The movement of liquid water across the eggshell of *Chelydra serpentina* eggs was assessed using a non volatile dye dissolved in the water used to wet the incubation medium. A liquid water connection seemed evident during early incubation, but was not present after day nine in either year of this study. Eggs consistently took up dye while losing water and failed to take up dye while gaining water. Therefore, the liquid connection was considered to be along the fibers of the eggshell membrane and no filling the pores of the membrane and allowing bulk liquid water flow. The main water exchange mechanism throughout incubation was considered to be a vapor pathway.

Introduction

Reptile eggs exchange water with their environment during incubation (Ratterman and Ackerman 1989; Tracy and Snell 1985; Kam and Ackerman 1990; Tracy, Packard, and Packard 1978; Packard and Packard 1984; Ackerman et al. 1985b; Packard et al. 1979a;
Packard, Packard, and Boardman 1981). This water must move across the reptile eggshell which will also serve the respiratory requirements of the developing embryo.

The mechanism by which water is transported between the egg and the environment remains controversial (Ackerman, Seagrave, Ar, and Dmi’el 1985a; Tracy, Packard, and Packard 1978; Thompson 1987) but seems likely to be of some importance if we are to understand eggshell function. The movement of water across the eggshell has been characterized as a vapor transport process (Ackerman, Seagrave, Ar, and Dmi’el 1985a; Ackerman 1994) or as a liquid transport process (Tracy, Packard, and Packard 1978; Thompson 1987). The distinction between vapor and liquid water transport is important because of the respiratory gas exchange also occurring across the eggshell. The presence of liquid water transporting across the shell will effect oxygen exchange across the eggshell due to the very low solubility of oxygen in water compared to air (Kutchai and Steen 1971; Wangensteen Wilson and Rahn 1970/71; Lomholt 1976; Kayar et al. 1981). If water occludes air filled pores in the eggshell, eggshell conductance to oxygen will be reduced by 20 to 30 fold. On the other hand, if water is transported solely in the vapor phase, the exchange of respiratory gases and water can occur within the same gas filled pores and will be 20 to 30 fold greater than if the pores are filled with water. The primary objective of this paper is to assess to what extent liquid water or vapor water acts as the transport mechanism for water across the reptile eggshell.
Materials and Methods

Source and Condition of Eggs

All eggs were obtained from Millard’s Turtle farm in southeast Iowa. The eggs were a by-product of the turtle slaughter industry. The eggs were harvested as nesting female snapping turtles (*Chelydra serpentina*) were captured and slaughtered for their meat. The eggs were collected by removing the oviducts containing eggs from the carcass. The oviducts were then placed between moist cloth towels inside of a cooler for transport to our laboratory. This procedure assured that the eggs were in contact with fluid in the oviducts until they were used in the experiment.

Incubation Procedure for All Eggs

All containers used to incubate eggs were weighed, filled with sand, and weighed again. The mass of the sand was then calculated by subtraction and sufficient water was added to bring the water content of the sand to 4% by mass. The water potential associated with this water content in sand was -7 kPa (Kam and Ackerman 1990). The mixture of water and sand was transferred to a plastic bag where it was shaken to assure an even distribution of water in the sand and returned to the original container and the mass recorded. The mass of an egg subsequently placed in the container was added to obtain the total mass of each container. Weekly throughout incubation the mass of each container
was assessed for water loss and distilled water was added to maintain the mass of the containers at their original values, after taking into account water uptake by eggs.

All eggs were fully buried in the substrate at a depth of 2.5 cm to insure that the substrate water potential was constant throughout incubation (Kam and Ackerman 1990).

Experimental Procedure for Fluorescein Experiments

Ten *Chelydra serpentina* females with 24 or more eggs were chosen for this experiment. If more than 24 eggs were present, 24 eggs were chosen at random from the clutch by use of Procedure Plan in SAS (1990) with factors equal to the number of eggs in the entire clutch. Each egg was assigned a random number from 1 to 24 using Procedure Plan in SAS (1990) with factors equal to 24. The eggs assigned numbers 1-3 were weighed and immediately incubated in sand containing a 0.1 Molar fluorescein sodium salt \((C_{20}H_{10}Na_2O_4)\) solution. The fluorescein sodium solution was used to bring the water content of the sand used for incubation to 4% by mass. Fluorescein was used as an indicator of liquid water flow because it is a non-volatile dye and therefore should not traverse the shell and shell membrane in the vapor phase (Thompson 1987). The eggs remained in the experimental container for 48 hours. After 48 hours, the eggs were removed from the experimental substrate and weighed. They were then sampled for the presence of fluorescein using a 23 gauge needle attached to a 3 cc syringe. Thin albumin was withdrawn from each egg being careful not to contaminate the sample with dye that adheres to the outside of the eggshell. Eggs numbered 4-24 were weighed and placed into
individual cups and remained in these until they were moved into the experimental setup. Eggs numbered 4-6 were then weighed and placed into the experimental setup on day 3 and removed for weighing and sampling 48 hours later. Three eggs were also introduced into the experimental setup for 48 hours on days 6, 9, 12, 15, 18, and 21. On occasion the females used in 1994 had more than 24 eggs, so an extra twelve eggs were used on days 6, 12, and 18. Additionally, eight extra eggs from various other females were used on days 24 and 56.

The samples of thin albumin were tested for the presence of fluorescein using a spectrofluorometer (SLM AMINCO, model number 500C). The samples were scanned with the excitation wavelength set at 495 nm and the emission wavelength set at 530 nm. The samples were scanned from 400-600 nm and each of these scans was retained for further assessment of the presence of fluorescein. In addition to the scan, the reading at 495 nm was also recorded and was used as a measure of the relative amount of fluorescein present in the sample (figure 1). The weight of eggs before and after the exposure to fluorescein was used to calculate the quantity of water exchanged during the experiment.

Experimental Procedure for Eggshell Water Content Experiment

Six *Chelydra serpentina* females with 16 or more eggs were chosen for this experiment. If greater than 16 eggs were present, 16 eggs were chosen at random from the clutch by use of Procedure Plan in SAS (1990) with factors equal to the number of eggs in the entire clutch. Each egg was assigned a random number from 1 to 16 using Procedure
Plan in SAS (1990) with factors equal to 16. The eggs assigned number 1 were weighed and immediately opened and the contents emptied so the wet mass of the eggshell could be determined. The empty eggshell was weighed wet, dried and weighed again to determine the water content of the eggshell. The remaining eggs numbered 2-16 were incubated in the individual cups until used. Each day for the first 10 days of incubation, eggs were removed sequentially to determine the water content of the eggshell. Eggs were cleaned of debris by using a paint brush and immediately weighed. Then if the opaque patch had begun to spread, the egg contents were removed and the opaque and translucent fractions were separated so a determination of opaque fraction could be determined. Any materials that were adhering to the inside of the eggshell were carefully removed. The eggshell fractions were quickly weighed and then placed in an oven at 105 C for 24 hours with a loose fitting cover. After 24 hours, the eggs were removed from the oven and allowed 10 minutes to cool with the loose cover still in place. The eggs were then weighed again. The water content of each fraction of the eggshell was then determined using the wet and dry mass of the eggshell. If the opaque patch had not yet appeared the egg was treated in the same manner as the eggs used on day 0. That is, they were opened, emptied, the eggshell weighed, then dried, and reweighed. The percent of the eggshell that had dried was determined by dividing the dry weight of the top by the total eggshell dry weight. The dry fraction of the opaque part of the shell was expressed as a fraction of the whole eggshell dry mass. Additionally, the water content of the entire eggshell was also calculated by a
combination of the wet and dry weight for both the translucent and the opaque eggshell fractions.

Statistical Design

The SAS (1990) statistical package was used to test for differences between treatments. For this experiment General Linear Models (GLM) were used to set up the tests of significance because of missing values. For mass change in 48 hours, fluorescein readings, percent of eggshell that had become opaque, and water content of the entire eggshell the models were essentially the same. Each of these values were considered the dependent variables and the main effects of females and the main effects of the day the eggs were used or the day the eggs were placed into the experimental setup were tested. The error term for each of these was the female by day interaction.

To determine if differences between opaque and translucent eggshell fractions existed a new variable called eggshell fraction was created. Fraction was labeled 0 for the opaque fraction and 1 for the translucent fraction of the eggshell. The model used water content of the eggshell as the dependent variable and tested the main effects of eggshell fraction and female, while using eggshell fraction by female interaction as the error term.

Results

In both 1994 and 1995, the eggs exposed to fluorescein demonstrated a similar pattern of uptake. Eggs used on days earlier than day 10 generally had fluorescein readings
higher than 3, while eggs used after 10 days of incubation had readings that averaged close to 0 (figure 2). Because differences exist in the variance of the fluorescein readings before and after day 10, the combined Standard Error of the Mean (SEM) for days earlier than day 10 was computed separately from the SEM for days later than day 10. The day the eggs were used in the experiment was a significant factor in determining the amount of fluorescein that would be taken up in both years (p <.0001). The quantity of fluorescein taken up was dependent upon the female used in 1995 (p =.0129) but not in 1994 (p =.3858). The fluorescein readings from eggs used in 1994 and 1995 were not statistically different (p =.8135).

Eggs exposed to fluorescein for 48 hours changed in mass, some increasing in mass and some decreasing in mass (figure 3). If eggs were used early in incubation they often lost mass during the 48 hours of incubation (figure 3), while older eggs typically increased in mass. In 1994, eggs lose mass as late as day 21, while in 1995 no eggs lose mass after day 6 (figure 3). When the mass change is compared between years, the differences among years is significant (p =.0091). The pattern of mass change was significantly different among the days the eggs were used for both 1994 (p <.0001) and 1995 (p <.0001). The differences among females were significant in 1994 (p <.0001), while differences among females in 1995 were not (p =.0995). The mass change patterns for each year, upon inspection, seem to be somewhat linear (figure 3). When each year is tested for a linear component, a clear linear representation can be made for 1994 (p <.0001) and 1995 (p <.0001) (figure 3). Additionally, the slope of the linear component is different from zero
in both 1994 (p = .0002) and 1995 (p < .0001). The data for 1994 does not include day 56
data for computing or testing the linearity of the relationship between the day an egg is
used in an experiment and the mass change over 48 hours. Water exchange at day 56 is
believed to be represented by another linear relationship or possibly an exponential
relationship due to eggshell expansion that has occurred as a result of previous water
uptake. The quantity of fluorescein present in the egg after the experiment is shown as a
function of egg mass change during the experiment in figure 4.

The eggs that were obtained directly from the turtle’s oviducts were entirely
translucent indicating the presence of fluid in the pores. As incubation progressed an
opaque patch started at the top of the egg and spread to cover the upper half of the egg
(figure 5). During the first 6 days of incubation a successively larger fraction of the
eggshell changes from translucent to opaque. After the initial 6 days of opaque patch
spreading the percentage of area that is opaque remains constant. The day an egg is
sampled is highly significant in determining the percent of eggshell covered by the opaque
patch (p < .0001). Additionally each female’s eggs tended to dry to nearly the same
percentage and therefore the female effects are significant as well (p < .0001).

When the water content of the opaque fraction of the eggshell is compared to the
water content of the translucent fraction of the eggshell, the translucent fraction had a
higher water content in nearly every case (figure 6). The difference in water content
between the opaque fraction and the translucent fraction are nearly constant throughout
incubation and this difference is significant (p < .0001). For each female the difference
between the opaque fraction of the eggshell and the translucent fraction of the eggshell was nearly the same, but the water content values were different among females. Therefore female differences are also significant (p<.0001).

When the entire eggshell water content is determined, a pattern of early water content decline of approximately 10% is observed (figure 7). The day an egg is used plays a role in determining the water content of the entire eggshell and this is significant (p<.0001). Additionally, the differences between females are still present and these differences are significant (p<.0001).

Discussion

Eggshell Drying

When a turtle egg is laid by a female, the eggshell is fully saturated with fluid. The eggshell needs to lose at least some water in order to maximize respiration. The water content of the entire eggshell has been measured over the course of incubation (figure 7). The water content declines initially then remains relatively constant throughout incubation as the opaque patch spreads over the surface of the eggshell. The white patch has been characterized by Thompson (1985) for a tortoise eggshell and by this experimenter for *Chelydra serpentina* eggs and may cover from 60-100% of the eggshell surface (figure 5). The decrease in total eggshell water content and spreading of the opaque patch are closely paralleled. The water content associated with the opaque patch, as well as, the translucent
portion of the eggshell are relatively constant with time (figure 6). How the water is
removed from the shell membrane is unknown in turtle eggs. In bird eggs there is
controversy over this issue. Kutchai and Steen (1971) have postulated that the loss of
water from the albumin increases the colloid osmotic pressure and this draws water in from
the membranes. This data has been supported by Lomholt (1976), and Tullett and Board
(1976) using avian eggs, as well as, by Feder et al. (1982) on Chelydra serpentina eggs.
On the other hand, Kayar et al. (1981) suggest that the water is evaporated to the
atmosphere and is not drawn into the egg. Seymour and Piiper (1988) show evidence in an
avian egg that supports the drying of shell membranes from the inside. Additionally,
drying of the egg by evaporation including some involvement of the chorioallantois is not
supported because the chorioallantois does not reach the inner shell membrane until after
much of the drying process is over (Seymour and Piiper 1988). This is very similar to the
process observed in turtle eggs. Most importantly, the water is removed from the eggshell
enabling respiration to occur.

Transportation of Water

The pattern of fluorescein uptake in figure 2 may be taken as evidence that the
eggshell has the capacity to transport liquid water during the early stages of incubation.
The pattern observed supports the contention that a liquid water connection across the
eggshell exists early in incubation. This is consistent with the conclusions of Thompson
(1987) but his experimental results were ambiguous. The experiment used freshly laid
eggs, half buried in sand with fluorescein sodium present in the sand to monitor liquid water flow. During a 48 hour period, the dye had crossed the shells of the eggs, demonstrating that a liquid water pathway existed under the experimental conditions. The use of freshly laid, still wet eggs ensured that the fully saturated pores of the eggshells were in contact with the substrate. Secondly, the eggs in this experiment all lost water. The liquid water path depicted between the eggs and the environment is, therefore, not easily visible. The dye was moving one direction and the water was moving in the opposite direction. Additionally, the experiment was only conducted during the first two days of incubation. Therefore, the liquid connection reported may only exist for a short period during early incubation. More importantly, the conclusions of this paper, which argues for a liquid water connection throughout incubation are untested. The use of this data is therefore limited strictly to the discussion of the first two days of incubation.

The mass change over a 48 hour time period for different days of incubation in figure 3 indicates an increase in water uptake as incubation progresses. The data from 1994 at day 56 further this point by suggesting an exponential water uptake as incubation enters its later stages. The movement of water in the liquid phase would therefore bring with it an influx of fluorescein. On the contrary, the fluorescein levels are seen to fall to levels near zero and remain so after the twelfth day of incubation (figure 2). This would suggest that water movement and fluorescein uptake are independent.

Additionally, the idea of fluorescein movement and water movement being independent in this experiment is exemplified by figure 4. More important than the lack of
a relationship between water uptake and fluorescein uptake is that the movement of fluorescein is never associated with an influx of water. This result is consistent with the findings of Thompson (1987), as all of his eggs in contact with the substrate took up fluorescein and lost mass. Two factors could account for these findings. First the water movement could be primarily in the vapor phase as predicted by Ackerman, Dmi'el, and Ar (1985a), therefore yielding mass gain with no movement of fluorescein. Secondly, the observed low level liquid water movement may not be water movement at all, but could be explained by a similar phenomenon found in avian eggshells. Water is held in the fibrous structure of the chicken eggshell membrane by absorption (Simons 1971; Wangensteen and Weibel 1982). The absorbed water forms a continuous network of aqueous channels that connects the entire shell membrane. This system of aqueous channels allows for the calcium flux between the eggshell and the chorioallantois (Seymour and Piiper 1988). The presence of fluorescein may not therefore represent a bulk flow process as suggested by Thompson (1987), but the slow trickle pathway of diffusion of fluorescein from high concentration to low concentration. The diffusion is via very small flooded pores or along the absorbed water within the fibrous network of the shell membrane. In either of these cases the liquid water is not moving into the egg because the pressures necessary to move water bound to the fibrous network or move water through these tiny pores is extremely high. Therefore if the water cannot move as a liquid via these pathways and eggs are to take up water, the water must move across the eggshell as a vapor. Additionally, because the water is in a vapor form the respiratory pathways would not be impeded. Therefore the
water transport between the environment and Chelydra serpentina eggs would likely be in
the form of vapor water transport for most of the incubation period.

Acknowledgments

We would like to thank Chris Caon and Jason Rose for aiding in the collection and
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experiments. Additionally, we extend our gratitude to Dr. Sheldon Shen for the use of his
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mineral layer to diffusion of oxygen and water in flexible-shelled eggs of the


Figure 4.
Figure 6.
Figure legends

Figure 1. The spectral scan for various levels of fluorescein present in samples.

Figure 2. The relative amount of fluorescein present in the thin albumin samples for eggs used on different days of incubation. The SEM for eggs used before day 10 is 1.28, with an $n_h$ of 9.39. The SEM for eggs used after day 10 is 0.014, with an $n_h$ of 9.92. The data are presented with one standard deviation used as error bars.

Figure 3. Mass change in 48 hours for eggs used on different days of incubation. The SEM for 1994 data is 0.021g, while the SEM for 1995 is 0.019g. The lines represent the best fit lines for each year separately. The line for 1994 data does not include day 56 data as explained in the text. The harmonic mean ($n_h$) of sample size used as $n$ for 1994 mass change data was 7.93, while the $n_h$ for 1995 mass change data was 9.73. The data are presented with one standard deviation used as error bars.

Figure 4. Fluorescein readings compared to the amount of water exchanged in 48 hours with 1994 and 1995 data combined. SEM for the fluorescein reading is 1.01, while the SEM for mass change in 48 hours is 0.052g. The $n$ for each of the SEM values is 2 because the mean data for 1994 and 1995 was used as data points in the analysis. The data are presented with one standard deviation used as error bars in each direction.
Figure 5. Percent of the eggshell that has dried throughout incubation averaged over six females with no female effects removed. The dry patch of the egg was separated from the translucent fraction. Both of these fractions were then dried and the dry weight of the opaque patch was expressed as a percent of the total dry mass. The data are presented with one standard deviation used as error bars.

Figure 6. The water content of the opaque fraction and the translucent fraction of the eggshell. The data are presented with one standard deviation used as error bars.

Figure 7. Water content of the entire eggshell throughout incubation from different eggs, no effects of different females have been removed. The data are presented with one standard deviation used as error bars.
EFFECTS OF MAINTAINING VARIOUS CONSTANT WATER POTENTIALS ON HATCHLING SIZE AND WATER EXCHANGE OF EGGS FROM SNAPPING TURTLES (*Chelydra serpentina*)

A paper to be submitted to Physiological Zoology

Todd A. Rimkus and Ralph A. Ackerman

Abstract

The effects of various constant water potentials over the length of incubation of *Chelydra serpentina* eggs were examined by setting the water potential of a substrate to a constant value by the use of a new method. The new method utilizes the combination of matric and osmotic water potential to generate constant water potentials that vary much less than prior methods. The water potentials used were -7, -107, -207, -307, -407, -607, -807, and -1007 kPa. Eggs took up the most water at -7 kPa. Water loss was observed at -1007 kPa. A size index was created to compare a suite of size variables with respect to water uptake and water potential. Variation due to females was highly significant in determining size and therefore female effects were taken out as a main effect so the main effect of treatments could be examined independent of female effects. The largest hatchlings did not come from eggs incubated in substrates with the highest water potentials. Even though the most water was taken up by eggs held on a water potential of
-7 kPa, the largest hatchlings were those incubated at -107 kPa. Eggs held on -107 kPa had
the second largest water uptake. The upper limit of yolk mobilization by increased water
uptake may have been exceeded. The smallest hatchlings came from eggs incubated at
-1007 kPa as expected.

Introduction

Water movement is governed by differences in total water potentials (Hillel 1982). The
direction of movement is always from the higher water potential to the lower water
potential. Therefore, differences between egg water potentials and the water potential of
the incubation environment will determine water uptake or water loss throughout
incubation. The water potential of turtle eggs has not been accurately measured. Work on
other reptile eggs has yielded results that suggest the water potential of reptile eggs to be
approximately -800 kPa (Muth 1981; Ackerman 1991).

Substrate moisture has been correlated to water uptake, where eggs incubated on
wetter substrates take up the most water (Miller and Packard 1992; Packard, Packard, and
Gutzke 1985; Cunningham and Huene 1938; Tracy, Packard, and Packard 1978; and
Packard 1991). Therefore, water potentials of the substrate in wetter environments must
have been higher than the water potential of the eggs.

Additionally, eggs incubated on wetter substrates produce the heaviest hatchlings
(Cunningham and Huene 1938; Tracy, Packard, and Packard 1978), but no mention of any
other size variables is made. Packard (1991) extends this statement to include all
laboratory studies on size. However, the water potential values associated with moist substrates are somewhat ambiguous.

In natural environments, hatchlings of various sizes emerge from the same nests (Hotaling et al. 1985). Additionally, experimental manipulation of natural nests to remove female effects yields results that show variation of size dependent upon location of nests, as well as, location within a nest (Packard, Miller, and Packard 1993). This study only involved two nests and no physical measurements were made to determine if differences were present between nest locations or locations within the nests, so the differences could not be assessed further.

Therefore the work presented here is designed to address the water uptake of eggs incubated at a variety of constant water potentials, as well as, to determine how the hatchlings are affected by the constant water potentials. The method for maintaining a constant water potential is new and therefore the reliability of the method will also be addressed.

Materials and Methods

Source and Condition of Eggs

All eggs were obtained from Millard’s Turtle farm in southeast Iowa. As female snapping turtles, *Chelydra serpentina*, were slaughtered for their meat, eggs were harvested. The eggs were transported by removing the oviducts containing eggs and
placing the oviducts between moist towels inside of a cooler. The eggs were therefore in
contact with oviduct fluid until they were to be used in the experiment.

Experimental Procedure

_Chelydra serpentina_ females with 14 or more eggs were chosen for this experiment.
If more than 14 eggs were present, 14 eggs were chosen at random from the clutch by use
of Procedure Plan in SAS (1990) with factors equal to the number of eggs in the entire
clutch. Each egg was assigned a random number from 1 to 14 using Procedure Plan in
SAS (1990) with factors equal to 14. Eight treatments were to be assigned to eight of the
eggs, while the remaining six eggs were to be used for a related experiment. The
treatments were a range of constant water potentials at -7, -107, -207, -307, -407, -607,
-807, and -1007 kPa. These values for water potential were used because they likely
encompass the values of water potential that reptile eggs are exposed to during incubation
(Muth 1981).

All containers used to incubate eggs were weighed, filled with oven dried sand, and
weighed again. The mass of the sand was then calculated by subtraction. The containers
were then labeled according to the treatment that was to be applied to the egg assigned to
that container. Containers that were to have sand at -7 kPa had a sufficient amount of
distilled water added to bring the water content of the sand to 4% by mass. The water
potential associated with this water content in sand was -7 kPa (Kam and Ackerman 1990).
Containers that would hold sand at -107 kPa had enough -100 kPa sodium chloride
solution to bring the water content of the sand to 4% by mass. Thus the osmotic water potential of -100 kPa and the matric water potential of -7 kPa brought the water potential in the container to -107 kPa. Each of the remaining treatments were obtained by using sodium chloride solutions with the appropriate osmotic water potential as the solution in sand to bring the water content to 4% by mass. The mixture of distilled water or salt solution and sand was transferred to a plastic bag where it was shaken to assure an even distribution of water or solution in the sand and returned to the original container and the mass recorded. The mass of an egg subsequently placed in the container was added to obtain the total mass of each container. Weekly, throughout incubation, the mass of each container was assessed for water loss and distilled water was added to maintain the mass of the containers at their original values, after taking into account water uptake or loss by eggs. After about one sixth of incubation (estimated as 11 days) had passed, eggs were removed from the experimental set up cleaned of adhering debris with a paint brush and weighed. They were then returned to the same container. The weighing process was repeated every 11 days until hatching. Eggs that were pipped before a weighing date were not weighed. At a predicted time interval just before hatching eggs were again cleaned weighed and returned to their containers. As before if an egg had pipped or hatched prior to the predicted hatch date no weight measurement was recorded. After hatching, the hatchlings with an intact yolk sac were weighed. This mass was recorded as hatchling mass. Hatchlings were then placed in approximately 2 cm of water for 3 full days to allow the carapace to straighten before the linear measurements were made. After 3 days the
hatchlings were measured. The carapace length, width, and height were recorded. The hatchlings were then sacrificed. The yolk sac was removed from the hatchling and the wet body mass and wet yolk mass were recorded. The body and yolk were then dried for 7 full days at 105 C, to remove all water and obtain the dry body and dry yolk mass.

Statistical Design

The SAS (1990) statistical package was used to test for differences between treatments on each of the size variables. For this experiment General Linear Models (GLM) were used to set up the tests of significance because of missing values. For hatchling mass with yolk, carapace length, carapace width, carapace height, yolk wet mass, yolk dry mass, body wet mass, and body dry mass the models were the same. Each of these size variables was considered the dependent variable and the main effects of females and the main effects of treatments were tested. The error term for each of these was the female by treatment interaction.

Since we were trying to assess size differences it was decided that a size index should be generated. This was accomplished by using the method of principle component analysis. This analysis allowed us to examine the effects of treatments on overall size instead of on each of the size variables independently. In the event that female differences are significant the treatment effects and female effects need to be separated before principle components can be used. To accomplish this the MANOVA procedure is used in SAS (1990), this program is multivariate analysis of variance. The list of dependent variables is
inserted into the model statement and the “printh” and “printe” options produce sum of squares and cross product (SSCP) matrices for treatment effects and female effects, as well as, the error SSCP matrix. The matrices produced are then converted into correlation matrices by dividing each cell of the matrix by the square root of the product of the row and column diagonals. Principle components can then use the correlation matrix for treatment, female, and error effects separately to determine the greatest source of variation that exists in the individual matrices. The principle components are then ranked by the use of eigenvalues. The eigenvalues represent the percent of the total variation that can be explained by each of the principle components. Eigenvalues greater than one should be interpreted. The goal of principle component analysis is to find a latent variable which is a combination of some or all of the variables that were measured. The variables that we measured were all measurements of some aspect of size in and of themselves. The latent variable that we hoped to uncover was a variable that would represent overall size. If the individual variables contribute to the latent variable equally the variables can be standardized and their average used as the new size index variable, otherwise the value of the first principle component can be used as a weighting factor in producing the new variable. Standardization of the variables assures that all variables contribute equally with respect to magnitude of numbers. The water exchanged as a percent of initial mass and the new size index could then be treated as dependent variables where treatment effects and female effects could be tested and here the female by treatment interaction could be used for the error term.
Results

Water Exchange

The water exchanged by each egg was expressed as a percent of initial mass throughout incubation to simplify comparison between eggs (figure 1; figure 2). Eggs incubated at -7 kPa took up the most water throughout incubation (figure 1). The general trend present throughout incubation predicts that the higher the substrate water potential the higher the rate of water uptake (figure 1; figure 2). The water potential of -1007 kPa is the only water potential to yield a net loss of water during incubation (figure 2). The slopes of each water potential are presented in figure 3 and demonstrate that the greatest slope is associated with the highest water potential. The differences in slopes due to water potential differences are significant (p<0.0001), while the effect of female differences was not significant (p=0.6187).

Examination of total water exchange over the length of incubation shows an increase in water uptake as eggs are exposed to water potentials closer to zero (figure 4). Eggs held on -1007 kPa are the only eggs that demonstrate a total water loss over the length of incubation (figure 4). Additionally, eggs held on -807 kPa have a total water exchange closest to zero (figure 4).
Size Variables

Treatment significantly affected hatchling mass with yolk, carapace length, carapace width, carapace height, wet yolk, dry yolk, wet body, and dry body. The effects of coming from a particular female on each of these variables was of an even greater significance. The F values for effects due to female differences are greater for all variables except for the two variables measuring the yolk fraction (table 1). Since all variables were found to be significant, a principle component analysis was performed on these variables. The results of the principle component analysis yielded one principle component that accounted for 97% of the variability. The first principle component was approximately an equal weighting of each of the eight individual size variables. The yolk variables were of equal importance but their sign was opposite, that is in predicting size, yolk values are negatively correlated to the body and shell variables. Therefore, the latent variable was produced by adding the standardized body and shell variables while subtracting the yolk variables and dividing that sum by 8, the total number of individual size variables. The latent variable would then represent overall size as a size index.

The treatment effects on the new size index demonstrate that as the substrate water potential increases so does size. This was true for all treatments except that at the value of -7 kPa. The hatchlings from eggs incubated at a water potential of -7 kPa had a smaller size index than hatchlings from eggs held at water potentials of -107, -207, and -307 kPa (figure 5). The differences in size due to water potential differences are significant (p<0.0001), as are differences in the size index due to female differences (p<0.0001).
Table 1. The effects of treatments on size variables. Results for the individual size variables are presented with the F values associated with treatment and female effects. The probability of a greater F value is provided as well.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Source</th>
<th>F value</th>
<th>Probability of &gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>hatchling mass with intact yolk sac</td>
<td>Treatment</td>
<td>5.48</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>35.14</td>
<td>0.0001</td>
</tr>
<tr>
<td>carapace length</td>
<td>Treatment</td>
<td>8.19</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12.72</td>
<td>0.0001</td>
</tr>
<tr>
<td>carapace width</td>
<td>Treatment</td>
<td>7.17</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>8.63</td>
<td>0.0001</td>
</tr>
<tr>
<td>carapace height</td>
<td>Treatment</td>
<td>6.42</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>8.15</td>
<td>0.0001</td>
</tr>
<tr>
<td>wet mass of residual yolk</td>
<td>Treatment</td>
<td>9.11</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>3.12</td>
<td>0.0035</td>
</tr>
<tr>
<td>dry mass of residual yolk</td>
<td>Treatment</td>
<td>8.87</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>3.09</td>
<td>0.0038</td>
</tr>
<tr>
<td>wet mass of body without yolk</td>
<td>Treatment</td>
<td>8.04</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12.81</td>
<td>0.0001</td>
</tr>
<tr>
<td>dry mass of body without yolk</td>
<td>Treatment</td>
<td>9.89</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>11.69</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Additionally, size index was examined for effects due to the water exchange as a percent of initial mass (figure 6) and the results show an increase in size with water uptake. Again the result was true for all but the eggs that took up the most water, these were not the largest. The eggs that take up over 35% of initial mass are smaller than those taking up 15-25% of initial mass (figure 6).

Since yolk mobilization may play a role in determining size (Janzen et al. 1990). The mean and standard deviation are presented for yolk wet mass and yolk dry mass for
each constant water potential (table 2). The means for both yolk variables for the treatment of -7 kPa is larger than the means for treatments of -107, -207, and -307 kPa (table 2).

Discussion

Assessment of a New Method for Maintaining a Constant Water Potential

Water potential differences between the inside of the egg and the incubation environment indicate the direction of water movement in the event that water is able to move (Hillel 1982). Here we present a new method for establishing a constant water potential environment over the length of incubation, while maintaining the use of a natural substrate medium. We have chosen to fix the water potential in the substrate by combining two components of total water potential, matric water potential and osmotic water potential. Matric water potential is the pressure exerted by the capillary forces to hold water in the soil, also called matric suction (Hillel 1982). The matric water potential for 4% water in sand has been shown to be stable at -7 kPa over the length of incubation, as long as the eggs are at a depth below 2.6 cm in the incubation substrate and if water that evaporates from the upper surface is replaced approximately every 14 days (Kam and Ackerman 1990). This method of maintaining a constant water potential is likely only reliable down to about -25 kPa due to the shape of the characteristic curve for sands (Klute 1986; Ackerman 1991). In this range, the water content can be changed with little consequence in terms of changes in water potential. In order to produce stable water
Table 2. The mean and standard deviation for yolk wet mass and yolk dry mass.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Yolk wet mass (g)</th>
<th>Standard Deviation</th>
<th>Yolk dry mass (g)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>-7 kPa</td>
<td>0.85</td>
<td>0.56</td>
<td>0.37</td>
<td>0.28</td>
</tr>
<tr>
<td>-107 kPa</td>
<td>0.67</td>
<td>0.17</td>
<td>0.26</td>
<td>0.07</td>
</tr>
<tr>
<td>-207 kPa</td>
<td>0.67</td>
<td>0.34</td>
<td>0.29</td>
<td>0.18</td>
</tr>
<tr>
<td>-307 kPa</td>
<td>0.74</td>
<td>0.43</td>
<td>0.32</td>
<td>0.20</td>
</tr>
<tr>
<td>-407 kPa</td>
<td>0.88</td>
<td>0.61</td>
<td>0.40</td>
<td>0.30</td>
</tr>
<tr>
<td>-607 kPa</td>
<td>1.36</td>
<td>0.49</td>
<td>0.62</td>
<td>0.24</td>
</tr>
<tr>
<td>-807 kPa</td>
<td>1.78</td>
<td>0.56</td>
<td>0.83</td>
<td>0.28</td>
</tr>
<tr>
<td>-1007 kPa</td>
<td>2.63</td>
<td>1.55</td>
<td>1.14</td>
<td>0.66</td>
</tr>
</tbody>
</table>

potentials below -100 kPa in sand the water content would have to remain within 0.01% of its original value over the length of incubation. Small variations in water content could mean a change of 1000 kPa (Klute 1986). Therefore, the use of an osmotic water potential in conjunction with a matric water potential was envisioned. Osmotic water potential is dependent on a membrane being present in the system so differences in osmotic pressure can be generated across the membrane (Hillel 1982). In this experiment the eggshell membrane satisfies this requirement. The eggshell membrane will not allow salts to move across the membrane as long as only vapor water is transported across the membrane. This appears to be the case (See earlier). If liquid water were moving into these eggs the osmotic water potential differences would soon disappear as the salt concentration equilibrated between the inside and the outside of the egg, this was not observed. Additionally, the incoming salt would likely kill the embryos especially at higher salt
concentrations. Survival of hatchlings and water movement becoming less and less as more negative water potentials were encountered (figure 1 and figure 2) serves as additional support for water transport being highly dominated by the vapor phase throughout incubation. The final component of total water potential is gravitational water potential or the water potential dependent upon elevation in the soil column and can be omitted from the total water potential value we are using because the water and the egg will be in the same location with respect to the soil water column (Hillel 1982). Therefore, the water potentials given here are a combination of matric and osmotic water potentials. Matric water potential was the same for all containers and was obtained by keeping the sand at 4% water content by mass, this amount of water in sand is associated with a matric water potential of -7 kPa (Kam and Ackerman 1990). The total water potential is then determined by the amount of salt that has been dissolved in the water used to wet the sand. If the water used is distilled water the total water potential is -7 kPa, if the osmotic water potential of the salt solution used to wet the sand is -200 kPa the total water potential is -207 kPa.

are a number of problems associated with this method. Vermiculite is a clay particle found in low concentrations in many soil types but does not exist in a pure state in nature. The primary problem with vermiculite is its insulation properties. Heat produced by eggs incubating in vermiculite does not dissipate and therefore an unnatural heating is observed in these eggs (Kam and Ackerman 1990). The observed heating causes a loss of vapor and tends to be manifested late in incubation as metabolism increases the temperature of the eggs especially during late incubation (Getttinger et al. 1984). The increases in temperature cause the vapor pressure inside of the egg to increase the gradient in favor of diffusion of vapor out of the egg (Ackerman, Seagrave, Dmi’el, and Ar 1985; Packard et al. 1981a). In addition to the use of vermiculite as a substrate medium, most of these researchers incubate the eggs only half buried.

The method of half burying eggs came about to simulate eggs in contact with the nest and nest chamber. The half that is buried was considered to simulate that part of the egg that is in contact with the nest wall and the exposed half was to simulate an egg being exposed to the air space in the nest chamber. The problem with this method has to do with drying (Ackerman 1991). The surface of a soil will dry readily by evaporation or drainage. The boxes eggs are kept in by all who study turtle eggs lose mass over time. This loss is associated with evaporation and is a result of not being able to seal the boxes as oxygen exchange is necessitated (Lynn and von Brand 1945; Ackerman and Prange 1972; Ackerman 1977; Paganelli et al. 1978; and Ackerman 1980). Water is added to the boxes as much as twice a week. Therefore, the eggs in these boxes are exposed to a large range
of water potentials as the surface, where the eggs are, is drying and being wetted. The use of the term constant water potentials during incubation, therefore is not entirely true. The simulation of eggs exposed to the air space in a nest chamber is a potential catastrophe. Natural nest cavities are likely in equilibrium with the surrounding soils over much of the incubation period, where as water loss from the boxes is a clue that no such equilibrium is ever achieved in these experiments. It is the combination of heating and the inability to maintain the supposed equilibrium that likely causes the problem of late incubation water loss exhibited by the eggs used by many of these researchers. The loss of water during late incubation in these unnatural conditions may mask some of the treatment effects.

The eggs incubated in this experiment behave similarly to naturally occurring nests (Ratterman and Ackerman 1989; Packard and Packard 1984; Ewert 1985), and the embryos survive to hatching. This leads to the general conclusion that addition of salt to the incubation medium to maintain water potential has no readily observable adverse effects. This method of maintaining water potentials at constant values should replace the use of half buried eggs on the unnatural incubation medium vermiculite.

Analysis of Female and Treatment Effects

Differences in sizes of hatchlings has been attributed on many occasions to differences in incubation conditions, while ignoring the contribution of the female (Yntema 1976; Yntema 1978; Packard et al. 1981a; Packard et al. 1981b; Sarnat, McNabb, and Glass 1981; Gutzke and Paukstis 1983; Morris et al. 1983; Gutzke and Packard 1985;
Maternal effects are overwhelmingly important here and this supports the data that has taken female effects into account (Packard et al. 1981c; Packard et al. 1982; Bull, Vogt, and Bulmer 1982; Hotaling et al. 1985; Packard, Miller, and Packard 1993; Cagle et al. 1993). The maternal effects on size presented here play an even larger role than treatment effects in determining the individual size variables (table 1). The effects of females on determining the overall size index are also highly significant (p<0.0001; figure 5). Females effects are not important in determining the water exchange as a percent of initial mass presumably because the initial mass, which is largely due to female differences, is factored out by expressing water exchange on a per initial mass basis.

The water exchanged by eggs throughout incubation is consistent with reptile eggs being reported to have a water potential of about -800 kPa (Muth 1981; Ackerman 1991). The eggs incubated on substrates with a higher water potential than -800 kPa had mass increases at nearly all points during incubation (figure 1; figure 2), while eggs held on substrates with a water potential of -1007 kPa had a net mass loss over the course of incubation (figure 2). Finally, the treatment of -807 kPa resulted in only very small changes in mass over the entire length of incubation (figure 1). The data for individual slopes for treatments of -607 kPa and higher support mass uptake with time consistent with a lower water potential in the egg compared to the incubation environment (figure 3). Conversely, the negative slope associated with -1007 kPa assures that a net mass loss has occurred (figure 3). While the slope at -807 kPa is closest to zero (figure 3). Water
exchange as a percent of initial mass also supports the idea that the egg has a water potential near -800 kPa (figure 4). The greatest mass change is associated with the treatment, -7 kPa, that is furthest from the predicted -800 kPa value. Again -1007 kPa being lower than -800 kPa has a mass loss over the length of incubation (figure 4). The trend is nearly linear except the eggs held on -7 kPa seem to be shifted upwards (figure 4).

Other researchers do not see results that are entirely consistent with those presented here. Tracy, Packard, and Packard (1978) use a range of -200 kPa to -540 kPa on eggs from Chrysemys picta and find that mass of eggs change in similar ways except that a water potential of -540 kPa is roughly at equilibrium with respect to water balance throughout incubation, where as here we present mass uptake at -600 kPa. This may be the result of the different species used, but may also have to do with their eggs being half buried on vermiculite. No further analysis of size is performed on the hatchlings except to say that the hatchlings are heavier as the water potential of the incubation medium is increasingly higher. Many other studies have also demonstrated that water exchange is dependent upon hydric conditions of the incubating eggs (Brooks et al. 1991; Cagle et al. 1993; Packard, Packard, and Birchard 1989; Packard, Packard, and Gutzke 1985; Packard, Packard, and Boardman 1981a; Packard, Packard, and Boardman 1982b; Morris et al. 1983; Packard et al. 1983; Packard, Packard, Boardman, and Ashen 1981b; Packard, Packard, and Boardman 1984b; Packard et al. 1987). Again in these experiments, which use primarily -150 kPa as a wet substrate and -800 to -850 kPa as the dry substrate the eggs lose mass late in incubation independent of water potential encountered and a water
potential of -800 kPa is in a range of highly negative water balance for these eggs. The eggs in the previously mentioned studies are primarily from *Chelydra serpentina* and *Chrysemys picta*. Along with the influence of hydric conditions causing an effect on water exchange, size of resultant hatchlings was also found to be correlated with the hydric conditions.

The effects of treatments on the size index seem to follow the trend that the higher the water potential of the incubation environment the larger the hatchling (Brooks et al. 1991; Cagle et al. 1993; Packard, Packard, and Birchard 1989; Packard, Packard, and Gutzke 1985; Packard, Packard, and Boardman 1981a; Packard, Packard, and Boardman 1982b; Morris et al. 1983; Packard et al. 1983; Packard, Packard, Boardman, and Ashen 1981b; Packard, Packard, and Boardman 1984b; and Packard et al. 1987; Miller and Packard 1992; Packard 1991; Cunningham and Huene 1938; Tracy, Packard, and Packard 1978). In addition to the size of hatchling being greater on higher water potentials the yolk is decreased and this was a significant difference observed in our study as well (table 1; table 2). The trend of larger hatchlings from higher water potentials holds true for all treatments except -7 kPa (figure 5). Additionally, if size index is plotted against water exchange as a percent of initial mass by treatments the eggs which take up the most water are increasingly larger until the eggs which take up the largest amount of water are considered (figure 6). Here the eggs held on -7 kPa are seen to take up the most water but produce hatchlings that are smaller than eggs held on -407 kPa substrates (figure 6). This is the first evidence that an egg in a more favorable hydric environment with respect to
water exchange turns out to be smaller than the eggs held in the less favorable hydric environment. The hatchlings that result from eggs held on -7 kPa also have larger amounts of yolk than eggs held on -107 kPa to -307 kPa (table 2). Janzen et al. (1990) demonstrated that embryos held on a water potential of -150 kPa mobilized more lipids and proteins from the yolk than did embryos incubated on -850 kPa substrate. Embryos from eggs incubated on substrates with higher water potentials have also been shown to have higher metabolic rates (Gettinger et al. 1984). Here though, it seems that we have surpassed the upper limit and eggs gaining too much water may not be able to mobilize yolk as effectively once this upper limit has been reached.

The ecological impact of size differences in hatchling snapping turtles has yet to be fully determined. Brooks et al. (1991) found that temperature effects far outweighed and have longer lasting effects on fitness of hatchling turtles than does moisture, but they used vermiculite at -100 kPa and -500 kPa therefore reducing the magnitude of water potential differences usually used in laboratory studies. Packard, Packard, and Birchard (1989), as well as, Cagle et al. (1993) found that water potential has a greater influence on embryonic survival, mass of hatchlings, and size of hatchlings, than does temperature. The water potential in the former study was maintained constant using half buried eggs in vermiculite and so is not entirely reliable. The method used in the latter study on a natural nest is highly dependent on a constant temperature being present for an accurate reading and should not have been attempted where metabolic heating is likely to occur (Ackerman 1991). Differences in temperature on the order of 0.1 C can introduce an error of 1000 kPa
(Rawlins and Campbell 1986). The water potential differences observed by Cagle et al. (1993) may well be an artifact of the measuring system used.

Because the size index was generated by principle components using variables that necessitated termination of embryos the necessary measures of fitness are lacking from this study. The use of principle components in this study will allow further prediction of indices of size while only using a combination of hatchling mass, carapace length, carapace width, and carapace height. This will allow for the hatchlings to be used to measure performance so an assessment of fitness can be obtained (Arnold 1983).

Acknowledgments

We would like to thank Chris Caon and Jason Rose for aiding in the collection and care of the eggs used in these experiments. We would also like to extend our thanks to Fred Millard from Millard’s Turtle, who was more than willing to supply eggs for these experiments. Additionally, Fred Janzen was also very generous in supplying some of the eggs used in these experiments.

Bibliography


Figure 1.
Figure 2.
Figure 3.
Figure 4:

Water Exchange as a Percent of Initial Mass (%)

Water Potential (kPa)
Figure 5.
Figure 6.
Figure legends

Figure 1. Water exchange as a percent of initial mass for *Chelydra serpentina* eggs incubated on constant water potentials of -7 kPa, -207 kPa, -407 kPa, and -807 kPa. The eggs were weighed at 7 equal time frames throughout incubation. The data are presented with one standard deviation in the positive and negative direction.

Figure 2. Water exchange as a percent of initial mass for *Chelydra serpentina* eggs incubated on constant water potentials of -107 kPa, -307 kPa, -607 kPa, and -1007 kPa. The eggs were weighed at 7 equal time frames throughout incubation. The data are presented with one standard deviation in the positive and negative direction.

Figure 3. The slopes of the curves represented in figure 1 and 2. Each water potential value is nearest the line that represents eggs held at that water potential.

Figure 4. Total water exchange over the entire length of incubation as a percent of initial mass for the entire incubation period is expressed for each water potential value tested. The data are presented with one positive and one negative standard deviation.

Figure 5. The mean size index of hatchling from eggs held at different constant water potential values over the entire length of incubation. The data are presented with one positive and one negative standard deviation.
Figure 6. The mean size index is compared by the mean water exchanged as a percent of initial mass for the entire incubation time for each constant water potential. The data are presented with a positive and negative standard deviation for each mean.
EFFECTS OF PERIODIC SHIFTS IN WATER POTENTIAL ON HATCHLING SIZE AND WATER EXCHANGE OF EGGS FROM SNAPPING TURTLES (Chelydra serpentina)

A paper to be submitted to Physiological Zoology

Todd A. Rimkus and Ralph A. Ackerman

Abstract

The effects of periodic changes of incubation substrate water potentials was examined by changing the water potential of the incubation substrate for each third of incubation. The water potentials used were -7 kPa for a wet (W) substrate and -1007 kPa for a dry (D) substrate. The eggs could then be exposed to three doses of dry (DDD), two doses of dry (DDW, DWD, or WDD), one dose of dry (DWW, WDW, or WWD), or no dry doses (WWW). Water exchange was affected by the dose of water potentials, as well as, the period in which a particular water potential was encountered. Hatchling size was also dependent on dose, but the greatest effect was due to the substrate type encountered in the first period or first third of incubation. Influence of early incubation on hatchling size is important because if a female can assess moisture then site selection by females can have an effect on hatchling condition.
Water movement is governed by differences in total water potentials (Hillel 1982). The direction of movement is always from the higher water potential to the lower water potential. Therefore, differences between egg water potentials and the water potential of the incubation environment will determine water uptake or water loss throughout incubation. The water potential of turtle eggs has not been accurately measured. Work on other reptile eggs yielded results that suggest the water potential of reptile eggs is approximately -800 kPa (Muth 1981; Ackerman 1991).

Substrate moisture has been correlated to water uptake, where eggs incubated on wetter substrates take up the most water (Miller and Packard 1992; Packard, Packard, and Gutzke 1985; Cunningham and Huene 1938; Tracy, Packard, and Packard 1978; Packard 1991). Therefore, water potentials of the substrate in wetter environments must have been higher than the water potential of the eggs.

Additionally, eggs incubated on wetter substrates produce the heaviest hatchlings (Cunningham and Huene 1938; Tracy, Packard, and Packard 1978), but no mention of any other size variables is made. Packard (1991) extends this statement to include all laboratory studies on size. However, the water potential values associated with moist substrates are somewhat ambiguous.

In natural environments, hatchlings of various sizes emerge from the same nests (Hotaling et al. 1985). Additionally, experimental manipulation of natural nests to remove
female effects yielded results that demonstrate variation of size dependent upon location of nests, as well as, location within a nest (Packard, Miller, and Packard 1993). The Packard, Miller, and Packard (1993) study only involved two nests and no physical measurements were made to determine if differences were present between nest locations or locations within the nests, so the differences could not be assessed further. Additionally, eggs of *Chrysemys picta* have been exposed to different water potentials for different periods of incubation and assessed for effects of these changes on size (Gutzke and Packard 1986). Packard and Packard (1988) conducted very similar using *Chelydra serpentina* eggs and very different results were obtained in comparison to Packard and Gutzke (1986). The eggs used in both of these previous experiments lost water late in incubation due to using vermiculite as an incubation medium (Kam and Ackerman 1990). Therefore, eggs of *Chelydra serpentina* were examined using a similar protocol except eggs were incubated buried in sand instead of half buried on vermiculite.

**Materials and Methods**

**Source and Condition of Eggs**

All eggs were obtained from Millard's Turtle farm in southeast Iowa. As female snapping turtles, *Chelydra serpentina*, were slaughtered for their meat, eggs were harvested. The eggs were transported by removing the oviducts containing eggs and
placing the oviducts between moist towels inside of a cooler. The eggs were therefore in contact with oviduct fluid until they were to be used in the experiment.

Experimental Procedure

*Chelydra serpentina* females with 14 or more eggs were chosen for this experiment. If more than 14 eggs were present, 14 eggs were chosen at random from the clutch by use of Procedure Plan in SAS (1990) with factors equal to the number of eggs in the entire clutch. Each egg was assigned a random number from 1 to 14 using Procedure Plan in SAS (1990) with factors equal to 14. Eight treatments were to be assigned to eight of the eggs, while the remaining six eggs were to be used for a related experiment. The incubation period was divided into thirds, the water potentials were to be set at a wet (W) water potential of -7 kPa or a dry (D) water potential of -1007 kPa, and each treatment consisted of all possible combinations of shifts for this arrangement. The resulting treatments are: DDD, DDW, DWD, DWW, WDD, WDW, WWD, and WWW.

All containers used to incubate eggs were weighed, filled with oven dried sand, and weighed again. The mass of the sand was then calculated by subtraction. The containers were then labeled appropriately. The containers holding wet substrates had sufficient distilled water added to bring the water content of the sand to 4% by mass. The water potential associated with this water content in sand was -7 kPa (Kam and Ackerman 1990). The containers with sand at the drier substrate water potential had enough -1000 kPa sodium chloride solution to bring the water content of the sand to 4% by mass. Thus the
osmotic water potential of -1000 kPa and the matric water potential of -7 kPa brought the water potential in the container to -1007 kPa. The mixture of distilled water or sodium chloride solution and sand was transferred to a plastic bag where it was shaken to assure an even distribution of water or solution in the sand and returned to the original container and the mass recorded. The mass of an egg subsequently placed in the container was added to obtain the total mass of each container. Weekly, throughout incubation, the mass of each container was assessed for water loss and distilled water was added to maintain the mass of the containers at their original values, after taking into account water uptake or loss by eggs. After about one sixth of incubation had passed, eggs were removed from the experimental set up cleaned of adhering debris with a paint brush and weighed. They were then returned to the same container. When one third of incubation had passed, the eggs were again cleaned and weighed. The eggs that were to be shifted after the first third of incubation were shifted immediately after weighing. Eggs moving after the first third of incubation were eggs assigned to the following treatments: DWD, DWD, WDD, and WDW. Eggs remained in these containers until half of incubation had passed. At the half way point eggs were again cleaned and weighed and returned to their containers. After two thirds of incubation had passed, the eggs were cleaned and weighed and returned to their containers unless they were to be shifted. The treatments necessitating egg movement after two thirds of incubation had passed were as follows: DDW, DWD, WDW, and WWD. The shifts were made immediately after the eggs were weighed. After five sixths of incubation, eggs were cleaned, weighed, and returned to their appropriate containers. Eggs
that were pipped before a weighing date were not weighed. At a predicted time interval just before hatching eggs were again cleaned, weighed, and returned to their containers. As before, if an egg had pipped or hatched prior to the predicted hatch date no weight measurement was recorded. After hatching, the hatchlings were weighed with an intact yolk sac. This mass was recorded as hatchling mass. Hatchlings were then placed in approximately 2 cm of water for 3 full days to allow their carapaces to straighten. After 3 days the embryos were measured. The carapace length, width, and height were recorded. The hatchlings were then sacrificed. The yolk sac was removed from the hatchlings and the wet body mass and wet yolk mass were recorded. The body and yolk were then dried for 7 full days at 105 C, to remove all water and obtain the dry body and dry yolk mass.

Statistical Design

The SAS (1990) statistical package was used to test for differences between treatments on each of the size variables. For this experiment General Linear Models (GLM) were used to set up the tests of significance because of missing values. For hatchling mass with yolk, carapace length, carapace width, carapace height, yolk wet mass, yolk dry mass, body wet mass, and body dry mass the models were the same. Each of these size variables was considered the dependent variable and the main effects of females and the main effects of treatments were tested. The error term for each of these tests was the female by treatment interaction. Additionally, the effects of doses of dry or wet were compared by creating a new variable called dose. The dose variable was 0, 1, 2, or 3
dependent on the number of dry periods that an egg encountered. The main effects of dose and female were tested with each of the size variables as the dependent variable. The error term was female by dose interaction. Finally, the effects of each of the three periods of incubation, as well as female effects were tested with each of the size variables as the dependent variable. Interaction between all periods were also tested. The error term here consisted of interaction between period one, period two, period three, and female.

Because we were trying to assess size differences it was decided that a size index should be generated. This was accomplished by using the method of principle component analysis. This analysis allowed us to examine the effects of treatments on overall size instead of on each of the size variables independently. In the event that female differences are significant the treatment effects and female effects need to be separated before principle components can be used. To accomplish this the MANOVA procedure is used in SAS (1990), this program is multivariate analysis of variance. The list of dependent variables is inserted into the model statement and the "printh" and "printe" options produce sum of squares and cross product (SSCP) matrices for treatment effects and female effects, as well as, the error SSCP matrix. The matrices produced are then converted into correlation matrices by dividing each cell of the matrix by the square root of the product of the row and column diagonals. Principle components can then use the correlation matrix for treatment, female, and error effects separately to determine the greatest source of variation that exists in the individual matrices. The principle components are then ranked by the use of eigenvalues. The eigenvalues represent the percent of the total variation that can be
explained by each of the principle components. Eigenvalues greater than one should be interpreted. The goal of principle component analysis is to find a latent variable which is a combination of some or all of the variables that were measured. The variables that we measured were all measurements of some aspect of size in and of themselves. The latent variable that we hoped to uncover was a variable that would represent overall size. If the individual variables contribute to the latent variable equally the variables can be standardized and their average used as the new size index variable, otherwise the value of the first principle component can be used as a weighting factor in producing the new variable. Standardization of the variables assures that all variables contribute equally with respect to magnitude of numbers. The water exchanged as a percent of initial mass and the new size index could then be treated as dependent variables where treatment effects and female effects could be tested and here the female by treatment interaction could be used for the error term. Dose and incubation period were also tested for effects on the size index and water exchange as a percent of initial mass with the same design as used on the individual size variables.

Results

Water Exchange

The water exchange by each egg was expressed as a percent of initial mass to simplify comparison between eggs. The water exchange was analyzed by treatment, by
dose, and by period. The treatment effect on water exchange was significant (p<0.0001). The mean and standard deviation for differences due to treatments are presented in figure 1. The treatment of DDD was the only treatment that resulted in net water loss over the entire length of incubation (figure 1). The treatment WWW resulted in the highest water gain (figure 1). Water exchange was not affected by which female the eggs came from (p=0.0547). Next the differences in water exchange were examined with respect to dose. The dose was coded 0 for wet and 1 for dry. Thus the treatments WWD, WDW, and DWW would have a dose value of 1. The differences due to doses were significant (p=0.0002). The means and standard deviations of different doses is presented in figure 2. As dose of dry periods increased, the amount of water exchanged decreased and a dose of 3 resulted in a net loss of water (figure 2). Eggs from different females responded very similarly to the treatments by dose so differences due to females were still not significant (p=0.0656). Finally the water exchange was examined for each period. Each third of incubation was numbered as a period of incubation 1-3. Again if the dose called for a dry period the period was coded 1, and if it was to be a wet period of incubation the period was coded 0. Thus if the dose was WDW, period one and three were coded 0, while period two was coded 1. If period one was wet, the overall water exchange was higher than if it were dry (figure 3), but these differences were not significant (p=0.0610). The water exchanged in period two is significantly affected by being wet or dry (p=0.0342). The overall water exchange is 10 grams higher if eggs experience wet in period two than if they experience dry in that same period (figure 3). The third period is by far the greatest predictor for water
exchange and a full 20 grams more water is taken up by eggs incubated in wet substrates versus those incubated in dry substrates (figure 3) and these differences are significant (p<0.0001).

Size Variables

Hatchling mass with yolk, carapace length, carapace width, wet yolk, dry yolk, wet body, and dry body all had significant difference due to treatments (table 1). The only variable not exhibiting significant difference due to treatments was carapace height (table 1). All variables, whether affected by treatments or not, had a significant effect due to the female differences (table 1).

Next the data was examined to see if there was a dose effect on the size variables. The size variables affected by the dose are hatchling mass with yolk, carapace length, wet yolk, dry yolk, wet body, and dry body (table 2). Again all size variables are affected significantly by differences due to females (table 2).

Finally, the size data was further examined to see if the period during incubation that the egg experienced wet or dry conditions played a role in determining the size of the hatchling. For all size variables the effects of differences due to females were significant (table 3). Period one had significant effects on all size variables except hatchling mass with yolk (table 3). Size variables affected by being wet or dry in period two were hatchling mass with yolk, carapace length, wet yolk, dry yolk, wet body mass, and dry body mass (table 3). The effects of being wet or dry in period three had no effects on size
Table 1. The effects of treatments on size variables.

<table>
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<tr>
<th>Variable</th>
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<th>F value</th>
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variables (table 3). The interaction between any combination of periods had no effects on size either (table 3).

Since nearly all of the size variables were found to be significant for each of the analyses above, principle components was performed on these variables. The results of the principle component analysis determined that one principle component accounted for 91% of the variability. The first principle component was approximately an equal weighting of each of the eight individual size variables. The yolk variables were of equal importance but their sign was opposite, that is in predicting size, yolk values are negatively correlated.
Table 2. The effects of doses on size variables.

<table>
<thead>
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To the body and shell variables. Therefore, the latent variable was produced by adding the standardized body and shell variables while subtracting the yolk variables and dividing that sum by 8, the total number of individual size variables. The latent variable would then represent overall size as a size index.

The size index was affected by treatments (p=0.0153). The mean and standard deviation are presented in figure 4. Dose effects on the size index were also significant (p=0.0135). The mean and standard deviation for each dose are presented in figure 5. The effects on the size index by period are similar to what was observed earlier on each of the
Table 3. The effects of period differences on size variables.

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size variables, in that the period with the greatest effect is period one (p=0.0007). Period two did not have as large an effect as period one (p=0.0256). While, period three (p=0.8056) and the interactions between the periods (from p=0.1328 to p=0.7771) had no
effect on determining the size index. The results for the main effects of periods are presented in figure 6. Differences in the size index with respect to females was significant in the treatment ($p<0.0001$), dose ($p=0.0005$), and period ($p<0.0001$) analyses.

Discussion

Analysis of Female Effects

Differences due to females account for a large portion of the sizes that hatchlings attain independent of the water potential they are incubated on. Other studies that have assessed effects due to females found that female or maternal effects are very important as well (Packard et al. 1981c; Packard et al. 1982; Bull, Vogt, and Bulmer 1982; Hotaling et al. 1985; Packard, Miller, and Packard 1993; Cagle et al. 1993; Brooks et al. 1991). The genetic contribution and possibly the effects of female contribution to egg quality has frequently been dismissed. The effects of female differences was great in this experiment and bring up the possibility that the greatest influence on survivorship and fitness could be entirely based on parental contributions more so than on hydric conditions.

Analysis of Treatment, Dose, and Period Effects

Water potential differences will drive the movement of water in any system where water is able to move (Hillel 1982). If water is able to move it will always move from a higher water potential to a lower water potential. Reptiles lay eggs that have a water
potential around -800 kPa (Muth 1981; Ackerman 1991). Therefore, if the eggs are laid in a soil with a water potential more negative than -800 kPa the eggs will likely lose mass during incubation and conversely if they are laid on a substrate with a water potential less negative than -800 kPa the eggs will likely take up mass over the length of incubation (See earlier).

Soils that naturally incubate eggs are subject to wetting by precipitation or to drying by evaporation or drainage. Therefore the environment may or may not be constant in time (Ackerman 1991; Ratterman and Ackerman 1989; Packard, Paukstis, Boardman, and Gutzke 1985b). In the laboratory, eggs have been exposed to constant wet or constant dry environments throughout incubation (Janzen et al. 1990; Packard and Packard 1991; Brooks et al. 1991; Cagle et al. 1993; Packard, Packard, and Birchard 1989; Packard, Packard, and Gutzke 1985; Packard, Packard, and Boardman 1981a; Packard, Packard, and Boardman 1982b; Morris et al. 1983; Packard et al. 1983; Packard, Packard, Boardman, and Ashen 1981b; Packard, Packard, and Boardman 1984b; Packard et al. 1987; Miller and Packard 1992; Tracy, Packard, and Packard 1978). Water movement in these eggs has also been shown to be dependent on water potential of the constant incubation medium. Environmental conditions may change (Packard, Paukstis, Boardman, and Gutzke 1985b) and two studies have examined the idea of changing environments in the laboratory setting (Gutzke and Packard 1986; Packard and Packard 1988). Gutzke and Packard (1986) used Chrysemys picta eggs to study shifted water potentials and therefore any differences seen between their data and that presented here may ultimately be due to species differences.
Packard and Packard (1988) used *Chelydra serpentina* eggs, so direct comparisons between their data and that presented here can be made. Because differences exist between both of the previous studies and the data presented here, the differences may be due to the incubation of their eggs half buried on vermiculite (See earlier).

Water exchange as a percent of initial mass is different depending on the treatment (figure 1). The trend is consistent with a greater uptake with additional periods of wet substrate encountered. Figure 2 furthers this argument by looking at the data on a dose basis, where the dose of 3 dry periods results in eggs that lose mass relative to the other doses. Each additional dose of dry is seen to lower the overall water uptake. This is consistent with the results on painted turtle eggs (Gutzke and Packard 1986), as well as, the work previously performed on snapping turtle eggs (Packard and Packard 1988). Also somewhat consistent with the earlier results is the trend that early incubation has little effect on overall water exchange as a percent of initial mass (figure 3). Period two has a visible effect due in part to compensatory water exchange (Clark 1953; Gordon 1960; Gutzke and Packard 1986) and this is consistent with the findings of Gutzke and Packard (1986). Compensatory water exchange is also observed in the third period which was not evident in the *Chrysemys picta* data (Gutzke and Packard 1986).

The effects of water exchange on size have a fundamental discrepancy from the work on *Chrysemys picta* (Gutzke and Packard 1986), as well as, on *Chelydra serpentina* (Packard and Packard 1988). The greater number of wet periods encountered the larger the hatchling, is consistent with past reports (Gutzke and Packard 1986; Packard and Packard
However, the discrepancy is noted when period effects are examined (figure 6). The differences between sizes of animals is greatest for being wet or dry in period one. These effects are significant, yet Gutzke and Packard (1986) reported that period one shows no effects and period two is the most critical, while Packard and Packard (1988) show the effects of period two and three to greatly outweigh the effects of period one.

The differences may be due to species differences for Gutzke and Packard (1986), while Packard and Packard (1988) use the same species as we have. Another difference is in the methods used to incubate eggs. Gutzke and Packard (1986), as well as, Packard and Packard (1988) incubated eggs half buried on vermiculite. This experimental difference may help us to understand why differences in water exchange are reported and why there is the discrepancy in when compensatory water exchange is observed. The composition of vermiculite makes it a good insulator, which can have adverse effects on eggs causing water loss due to excessive heat build up during late incubation (Kam and Ackerman 1990; See earlier). The increase in insulation and heat will drive vapor water from the egg under conditions that would normally predict mass gain. This additional factor or the use of half buried eggs or both (See earlier) may contribute to mass loss at the end of incubation and act to mask the compensatory water exchange that was expected by Gutzke and Packard (1986).

The unnatural conditions of excess heating and water loss may have also hidden the effects of period one. The nest selection process by females, more specifically the searching behavior, has been observed (Packard, Taigen, Packard, and Boardman 1980). It
has been suggested that the female may be assessing moisture. However, the searching process would not be as important if the conditions of the nest chamber are likely to change during incubation (Packard, Paukstis, Boardman, and Gutzke 1985b) and period one has no effects on hatchling size. The data presented here suggest that period one is the most important period in predicting overall hatchling size independent of what environments are observed later. Therefore if a female assesses the environment for a favorable nesting site the hatchlings have received some benefit to overall size by being placed in a favorable site early in incubation, regardless of the conditions later in incubation.

Hatchling size has not been fully assessed in Chelydra serpentina, but larger Chelydra serpentina hatchlings have been observed to out compete smaller hatchlings for food (Froese and Burghardt 1974). Additionally, Evans (1940; 1952) has demonstrated that Chelydra serpentina dominance is affected by size of hatchlings as well as injected testosterone levels. The need for a study that involves an assessment of hatchling survival and fitness is great. Therefore, the future direction of this study should include a mark and recapture experiment to assess lifetime fitness of different sized hatchlings. This would identify if the differences in size are important to this issue or if some other variable should be measured.
Acknowledgments

We would like to thank Chris Caon and Jason Rose for aiding in the collection and care of the eggs used in these experiments. We would also like to extend our thanks to Fred Millard from Millard’s Turtle, who was more than willing to supply eggs for these experiments. Additionally, Fred Janzen was also very generous in supplying some of the eggs used in these experiments.

Bibliography


Water Exchange as a Percent of Initial Mass (%)
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure legends

Figure 1. Water exchange as a percent of initial mass for the entire length of incubation is presented for each treatment. The incubation time was divided into three equal parts, and the letters correspond to different water potentials. W for wet is -7 kPa and D for dry is -1007 kPa. The data are presented with one positive and one negative standard deviation.

Figure 2. Water exchange as a percent of initial mass for the entire length of incubation is presented for treatments averaged by dose. A value of zero for dose means the egg never encountered the -1007 kPa condition. A dose of 1 corresponds to one of the three periods being held on the -1007 kPa water potential substrate. The data are presented with one positive and one negative standard deviation.

Figure 3. Water exchange as a percent of initial mass for the entire length of incubation is presented for each level of a period. The incubation time was divided into three equal parts, and the letters correspond to different water potentials. W for wet is -7 kPa and D for dry is -1007 kPa. A treatment of W _ _ is the average of all treatments that were held on -1007 kPa substrate for the first third of incubation and would include WWW WWD WDW and WDD. The data are presented with one positive and one negative standard deviation.

Figure 4. Size index is presented for each treatment. The incubation time was divided into three equal parts, and the letters correspond to different water potentials. W for wet is -7
kPa and D for dry is -1007 kPa. The data are presented with one positive and one negative standard deviation.

Figure 5. Size index is presented for treatments averaged by dose. A value of zero for dose means the egg never encountered the -1007 kPa condition. A dose of 1 corresponds to one of the three periods being held on the -1007 kPa water potential substrate. The data are presented with one positive and one negative standard deviation.

Figure 6. Size index is presented for each level of a period. The incubation time was divide into three equal parts, and the letters correspond to different water potentials. W for wet is -7 kPa and D for dry is -1007 kPa. A treatment of W _ _ is the average of all treatments that were held on -1007 kPa substrate for the first third of incubation and would include WWW WWD WDW and WDD. The data are presented with one positive and one negative standard deviation.
GENERAL CONCLUSIONS

The functional significance of the reptilian eggshell is likely a mechanism for uncoupling environmental liquid water from the liquid water within the egg. Egg water exchange is independent of the liquid water marker, that is, as mass increases occur the dye does not cross the eggshell. Therefore, it can be concluded that if liquid water does cross the eggshell, it does so early in incubation. As incubation progresses, the only avenue for liquid water becomes the liquid water adsorbed to the eggshell membranes. Water along this pathway can move but only very slowly compared to if the pores were liquid filled. The process of bulk flow plays no role in water exchange for reptile eggs. The rates of water vapor movement far outweigh any other water movement avenue over the entire length of incubation.

Water exchange plays a role in determination of hatchling size. This data has shown for the first time that animals encountering the wettest conditions do not attain the greatest size. The reason this finding has come about is likely due to the lack of testing more than two water potentials during an experiment and calling one wet (usually around -150 kPa) and the other dry (usually -700 to -1000 kPa). The effects of water potentials on water exchange and on size relationships other than for the condition where the most water is available show clear correlation between water potential, water exchange, and size. In the past, these relationships may have been somewhat obscured due to incubating eggs half buried in vermiculite. The upper limit of water exchange may have been exceeded in this experiment as embryos may have been unable to use the yolk effectively due to the
additional water that was present in eggs incubated on -7 kPa substrates. Additionally, the effects of periodic water availability show that water availability during early incubation is very important and therefore site selection by females may very well influence the fitness of her hatchlings. Again, past experimentation was likely confounded by the use of eggs half buried on vermiculite. Therefore, the conclusions covering water exchange and size point out that the use of unnatural incubation media may obscure or hide the true relationships, and eggs half buried on vermiculite for the most part should be abandoned.

The great contribution of female effects to this work and previous work should not be ignored. The differences due to females bring up the possibility that the greatest influence on survivorship and fitness could be entirely based on parental contributions more so than on hydric conditions or other environmental contributions.

Future directions of research should include furthering the size studies by including species with different shell types. The ease of movement of water for any given water potential difference will depend on eggshell type. Turtles have two shell types: parchment shelled and hard shelled. The parchment shelled eggs have a soft leathery eggshell that is characteristic of Chelydra and Caretta, as well as others. Hard shelled eggs are common in Apalone and others. The hard shelled eggs are similar in construction to bird eggs. The parchment shelled eggs have a greater number of pores than the hard shelled eggs (Packard and Packard 1980). The rate of water exchange in the parchment shelled eggs is, therefore, higher than the rate of exchange in hard shelled eggs (Ackerman et al. 1985b; Packard et al. 1979b; Packard, Packard, and Boardman 1982a; Packard, Taigen, Packard, and
Boardman 1981c). Hard shelled eggs of *Apalone*, observed under natural conditions, lost mass during incubation (Leshem and Dmi’el 1986). Similarly, losses have been observed in the laboratory environment (Packard et al. 1979a; Packard et al. 1981c). The water potential reported in the natural nest was -2760 kPa (Leshem and Dmi’el 1986). This water potential would predict the observed mass loss. Some of the water potentials used by Packard et al. (1979a), however, would predict mass gain. The cause for the observed losses may be due to the use of eggs half buried on vermiculite (Kam and Ackerman 1990), or perhaps the hard shelled eggs of reptiles may need to lose mass during incubation. This would be similar to what happens in the hard shelled eggs of birds. Hard shelled eggs of birds must lose about 15% of their mass in order to assure proper development (Davis et al. 1988; Lundy 1969). Water exchange is affected by the eggshell type (Ackerman et al. 1985b; Packard et al. 1979b) and hatchling size seems to be affected by water uptake (Gutzke and Packard 1986; Packard and Packard 1988; See earlier). The combination of these two ideas, a comparison of the effects of different shell types on water exchange and those effects on hatchling size, should also be investigated further. The current literature states that hard shelled eggs are environmentally insensitive (Packard, Packard, and Boardman 1982a; and Packard, Taigen, Packard, and Boardman 1981c). There is a need to measure the environment and use biologically relevant water potentials. This dissertation includes a method to accurately set water potentials using salts and these methods should be employed to avoid large fluctuations in water potentials with small changes in water content, a likely result if eggs continue to be half buried on vermiculite. Any additional
field data is likely to yield that conditions in nature are more adverse for this egg type than the levels of stress that have been placed on these eggs thus far under laboratory conditions.

Finally, hatchlings from *Chelydra serpentina*, snapping turtles, should be tested further in order to assess fitness based first on relating some performance factors to individuals then on determining fitness by some other method (Arnold 1983). This could include a mark and recapture study. The recent use of tagging devices in dogs includes one method that could be of value to many in this field. Small encoded metal bars are inserted under the skin which makes them easily identifiable based on reading the internal tag with a scanner. The use of this type of tag could be incorporated into a mark and recapture study on turtles. By inserting the tag in hatchlings prior to release one could monitor their lifetime fitness by recapturing them at a later date.
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