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CHEMICAL COMPOSITION OF THE EGGS OF THE FRESHWATER TURTLE *PHRYNOPS HILARII* (CHELIDAE: TESTUDINES)

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Abstract.—At oviposition, amniote eggs contain all the nutrients required for complete neonate tissue development. For reptiles and birds, the egg yolk is the main embryonic energy source and is composed predominantly of proteins and lipids, while also providing essential inorganic ions including calcium (Ca), potassium (K), and magnesium (Mg), and trace elements such as zinc (Z) and iron (Fe). Despite the fact that the Hilaire's Toadhead Turtle, *Phrynops hilarii*, is one of the most representative and widely distributed turtle species in Argentina, there are very few studies regarding its reproductive biology. The objectives of this work were to (1) describe the morphological characteristics of *Phrynops hilarii* eggs, specifically egg and yolk size; (2) determine the relative proportions of egg yolk components (water, ash, lipids, proteins, and minerals); and (3) examine the relationships between egg size, yolk size and yolk components. We collected 171 eggs from 11 individual nests during the 2012 March–April reproductive season. We analyzed one egg per clutch ($n = 11$) to determine the egg yolk chemical composition. Yolks were composed of 58.3% water, while the dry component was 2.7% ash, 24.9% lipids, and 56.7% protein. The most abundant macromineral detected was K (0.63%) and the most abundant trace mineral was Mg (0.13%). We did not find associations between egg measurements and components, but there were many significant correlations between egg components. Data we collected for this study are important to begin to understand the roles of different nutrients in embryonic development and to further explore maternal investment patterns in *Phrynops hilarii*.

Key Words.—egg yolk; lipids; minerals; proteins; turtles

Resumen.— En el momento de la oviposición los huevos amniotas contienen todos los nutrientes necesarios para el desarrollo completo del tejido neonatal. Para reptiles y aves, la yema del huevo es la principal fuente de energía embrionaria y está compuesta predominantemente de proteínas y lípidos y además proporciona iones inorgánicos esenciales que incluyen calcio (Ca), potasio (K) y magnesio (Mg) y trazas de oligoelementos como zinc (Z) y hierro (Fe). A pesar de que *Phrynops hilarii* es una de las especies de tortugas más representativas y ampliamente distribuidas de Argentina, existen muy pocos estudios sobre su biología reproductiva. Los objetivos de este trabajo fueron (1) describir las características morfológicas de los huevos de *Phrynops hilarii*, específicamente el tamaño del huevo y la yema; (2) determinar las proporciones relativas de los componentes de la yema de huevo (humedad, ceniza, lípidos, proteínas y minerales); y (3) examinar las relaciones entre el tamaño del huevo, el tamaño de la yema y los componentes de la yema. Recolectamos 171 huevos de 11 nidos individuales durante la temporada reproductiva de marzo a abril de 2012. Analizamos un huevo por nidada ($n = 11$) para determinar la composición química de la yema de huevo. Las yemas presentaron un 58,3% de humedad, mientras que el componente seco fue un 2,7% de ceniza, un 24,9% de lípidos y un 56,7% de proteína. El macromineral más abundante detectado fue K (0,63%) y el oligoelemento más abundante fue Mg (0,13%). No encontramos asociaciones entre las mediciones y los componentes del huevo, pero hubo muchas correlaciones significativas entre los componentes del huevo. Los datos que recopilamos para este estudio son importantes para comenzar a comprender los roles de los diferentes nutrientes en el desarrollo embrionario y para explorar más a fondo los patrones de inversión materna en *Phrynops hilarii*.

Palabras Clave.—yema de huevo; lípidos; minerales; proteínas; tortugas

INTRODUCTION

In reptiles, parental care has been reported in more than 140 species (approximately 3% of known species; Huang 2006). Offspring of oviparous species that do not exhibit parental care after oviposition have fewer opportunities to survive (Congdon et al. 1983). In chelonians, there is no parental care of eggs during embryonic development (Bujes and Verrastro 2008). The amniotic egg provides to the embryo a suitable environment for its development and contains all the required organic nutrients for complete neonate tissue development (Palmer and Guillette 1991; White 1991; Lombardi 1998; Thompson et al. 2001). Reptile eggs exhibit a wide variability in shape and size (Iverson and Ewert 1991); dimensions can vary as a function of shape, either spherical or oblong, or based on the water, lipid, and protein content (Congdon and Gibbons 1990; Roosenburg and Dennis 2005). The egg yolk is the main energy source provided by the female to the developing embryo in birds and reptiles (Miller and Dinkelacker 2008) and is composed primarily of proteins and lipids (White 1991; Noble 1991). The lipids provide the most significant nutritive component, being chiefly composed of the triacylglycerols, which is 70% of the lipid portion and is a concentrated energy source that fuels embryonic development throughout the course of incubation period (Speake and Thompson 1999). Reptile eggs contain approximately twice as much proteins as lipids, which together make up more than 90% of the egg yolk dry matter (Thompson et al. 2000; Thompson and Speake 2004). The most important inorganic components are calcium (Ca), sodium (Na) and phosphorus (P), as well as trace elements such as magnesium (Mg) and iron (Fe), altogether constituting approximately 5% of the dry matter of a fresh egg (Thompson et al. 2000).

Chelidae is a family of turtles currently composed of 54 species from 17 genera, with broad distribution in South America, as well as parts of Australia, Papua New Guinea, and Indonesia (Pritchard 1979; Fritz and Havas 2007; Vitt and Caldwell 2009; van Dijk et al. 2012). Seven species, including the Hilaire's Toadhead Turtle (*Phrynops hilarii*) from four genera, are found in Argentina (Prado et al. 2012). *Phrynops hilarii* is the largest species of the genus (Pritchard 1979) and its geographic distribution includes areas of central and northern Argentina (Cabrera 1998, Richard 1999). According to Freiberg (1977), Gallardo (1982) and Cei (1993), this species in Argentina has only one reproductive season lasting from November to March; but we have observed a longer breeding season including one peaks from August-September (spring) and another from March-April (autumn). The offspring of both breeding cycles hatch in February-March (summer). These incubation periods mean that some

embryos remain in the nest for almost a year (Prieto 2015). Turtle embryos use several developmental arrest mechanisms, including diapause (Ewert 1991), to lengthen the incubation stage. Although the function of diapause is still unclear, it may help ensure survival of the embryo over the cool winter period (Ewert and Wilson 1996). Species such as *P. hilarii* experience two diapause events: the first, primary diapause, occurs in the female oviduct and terminates during oviposition, and secondary diapauses are post-oviposition (Booth 2000, 2002). While the adaptive value of diapause is currently under debate, the prevalent idea in the literature suggests that this mechanism lowers mortality caused by low winter temperatures or causes the progeny to emerge at a time more suitable for the survival of hatchlings (Spencer et al. 2001; Horne 2007; Miller and Dinkelacker 2008). Research indicates that the secondary embryonic diapause in this species is obligate and is initiated independent of environmental conditions (Prieto 2015).

Despite its importance, most aspects of *P. hilarii* biology have not been thoroughly studied. Contributions to their biology are confined to national and local wildlife inventories (Richard and Waller 2000; Leynaud et al. 2006; Prado et al. 2012), general scientific literature (Cabrera 1998; Richard 1999), veterinary studies (Pérez Ganeselli et al. 2005), and research regarding the general phylogeny and evolution of turtles (Abdala et al. 2008). Studies focusing specifically on its reproductive biology (Bager 1997; Souza Bujes 1998; Tortato 2007; Lopez et al. 2013) and embryology (Serrano 1977; Piña and Argañaráz 2003; Fabrezi et al. 2009; Páez 2015; Prieto 2015) are scarce. The objectives of this study were to (1) describe the morphological characteristics of *P. hilarii* eggs, specifically the egg and yolk size; (2) determine the relative proportions of egg yolk components (water, ash, lipids, proteins and minerals); and (3) examine the relationships between egg size, yolk size, and yolk components. In the present work we hope to further our understanding of the yolk composition, the relationship between these components and how they affect embryonic development and maternal investment patterns in *Phrynops hilarii*.

MATERIALS AND METHODS

Study site.—The study site was located on a lowland area on the alluvial floodplain of the Paraná River in northeastern Argentina (Entre Ríos and Santa Fe provinces). Vegetation in the area is a mosaic of wet savannahs, grasslands, subtropical dry forests, gallery forests, shrublands, and a wide variety of wetlands (rivers, streams, marshes, swamps; López et al. 2013) that are strongly shaped by the water level cycles of the Paraná River (Neiff 1990; Burkart et al. 1999;

TABLE 1. Mean (\pm one standard deviation [SD]) and range of the chemical composition of egg yolks of Hilaire's Toadhead Turtles (*Phrynops hilarii*) from the Paraná River in northeastern Argentina. Values are expressed based on 100 g dry egg yolk ($n = 11$).

Components	Mean \pm SD	Range
Lipids (g)	24.85 \pm 4.99	17.80–32.90
Proteins (g)	56.64 \pm 3.94	47.30–62.00
Ash (g)	2.71 \pm 0.96	1.27–4.39
K (mg)	626.76 \pm 195.22	348.7–966.5
Ca (mg)	532.04 \pm 77.31	415.8–649.4
Mg (mg)	127.05 \pm 24.45	95.09–157.1
Fe (mg)	6.38 \pm 1.67	4.18–9.90
Zn (mg)	8.41 \pm 1.79	5.52–11.19

Casco et al. 2005). The climate is temperate to warm (19° C mean annual temperature) and wet (1,126 mm mean annual rainfall), with precipitation concentrated in the spring (October to December; mean = 348 mm) and summer (January to March; mean = 425 mm; Rojas and Saluso 1987; Aceñolaza, P., W. Sione, L.P. Zamboni, H. del Valle, F. Kalesnik, E. Rodriguez, F. Tentor, and J.F. Gallardo Lancho. 2010. Evaluación del evento de incendios extraordinarios del año 2008 en el Complejo Litoral del Río Paraná. I Jornadas Nacionales de Tecnologías y Métodos para la gestión del territorio, I Taller Nacional de Especialistas en Geodesastres, Argentina. Available from <http://www.ceregeo.org.ar/descarga/2010/3Acenolaza%20et%20a1%202010-JGeo.pdf>. [Accessed 15 December 2016]).

Egg collection and measurement.—We collected 171 eggs from 11 individual *P. hilarii* nests during the 2012 March–April reproductive season. After collection, we immediately transported eggs to the laboratory where we rinsed them with water to remove adhering soil. We measured the wet mass to the nearest 0.0001 g using a Precisa 180A Electronic Balance (Precisa Gravimetrics AG, Dietikon, Switzerland), and length and width to the nearest 0.05 mm using a manual caliper. We randomly extracted one egg from each nest ($n = 11$) for analysis of the egg yolk components; we opened each egg with a scalpel, separated the yolk from the albumen, and recorded its mass.

Egg yolk composition.—We analyzed the chemical composition analyses of 11 eggs (one per clutch). We calculated dry mass taking into account yolk total solids. Gravimetrically, we determined the water content of egg yolks by measuring the mass change in 1.5 g samples dried in an oven at 50° C for 24 h (AOAC, 1995). Afterwards ash content was measured by the mass change in dry samples after combustion in a furnace at 550° C (Association of Official Agricultural Chemists [AOAC] 1995). We calculated the total amount of lipids

from a portion (15 ml) of the yolk that we extracted in an excess of chloroform-methanol (2:1, v/v) and then allowed to evaporate at 70–80° C for 2 h (Folch et al. 1957).

To determine protein content, we used the Kjeldahl method (AOAC 1995) by means of an automated Kjeldahl procedure (Tecator System Digestor BUCHI K-355; BÜCHI Labortechnik AG, Flawil, Switzerland). We calculated the protein content from the nitrogen values, using a conversion factor of 6.25, which is commonly used to calculate protein in eggs (Maynard and Loosli 1969). Yolk protein can be inferred using the Kjeldahl technique of nitrogen analyses under the assumption that most of the nitrogen in egg yolk is in the form of protein (Thompson et al. 2001). We determined mineral content by dissolving sample ashes in 5 ml of HCl (3.5 N) and subsequently adding distilled water to a total volume of 25 ml. We employed a Perkin Elmer A Analyst 300 spectrophotometer (International Equipment Trading Ltd., Mundelein, Illinois, USA) to analyze the concentrations of Ca, Mg, Fe, and Zn by atomic absorption and the concentration of K by atomic emission (AOAC 1995). We tested data for normality (Shapiro-Wilks modified) but the data did not meet this assumption (even after transformation) so we used non-parametric Spearman's Rank Correlation Coefficient to ascertain descriptive relationships between egg yolk components and egg mass. We performed statistical analyses using INFOSTAT for Windows 2017 (Di Rienzo et al. 2017) and considered statistics significant at $P \leq 0.05$.

RESULTS

Phrynops hilarii nests contained an average of 15.6 eggs per clutch (standard deviation = 3.5, $n = 11$), and the eggs had an average wet mass of 19.75 g (± 1.95 g, $n = 171$), length of 32.46mm (± 1.16 mm, $n = 171$), and width of 31.97 mm (± 1.27 mm, $n = 171$). The egg yolks had an average wet mass of 7.89 g (± 0.78 g, $n = 11$) and albumen average wet mass was 8.92 g (± 0.41 g, $n = 11$). Water content of the egg yolks averaged 57.2% ($\pm 4.6\%$; range, 43.8–66.4%). We found positive and significant correlations between egg length with egg width ($r_s = 0.52$, $P < 0.001$), egg mass ($r_s = 0.91$, $P < 0.001$), and egg yolk mass ($r_s = 0.68$, $P < 0.001$). Lipid, protein, and ash of eggs varied from 17.8–32.9%, 47.3–62.0% and 1.3–4.4%, respectively (Table 1). We did not find any significant correlations between egg measurements and components, but there were significant correlations among the egg yolk components (Table 2).

DISCUSSION

In this study we found that *Phrynops hilarii* eggs were almost perfectly spherical and within the smallest

TABLE 2. Pearson correlation coefficients between egg yolk components (n = 11) of Hilaire's Toadhead Turtles (*Phrynops hilarii*) from the Paraná River in northeastern Argentina. An asterisk (*) indicates a significant correlation ($P \leq 0.05$).

	Proteins	Lipids	K	Ca	Mg	Fe	Zn
Proteins	1.00	0.58	0.83*	0.82*	0.78*	0.26	0.69*
Lipids		1.00	-0.25	-0.76*	-0.36	-0.35	-0.77*
K			1.00	0.69*	0.88*	0.32	0.55
Ca				1.00	0.81*	0.25	0.81*
Mg					1.00	0.23	0.66*
Fe						1.00	0.56
Zn							1.00

size range for turtle eggs (Iverson and Ewert 1991). The egg dimension data gathered during the study are consistent with *P. hilarii* populations in Brazil, Uruguay, and other areas of Argentina (Bager 1997; Cabrera 1998; Carreira et al. 2005; Bujes and Verrastro 2009). Egg size and components were not significantly correlated to each other, as is generally assumed in studies of the reproductive ecology of oviparous species (Roosenburg and Dennis 2005). Embryonic and postnatal survival, as well as hatchling fitness in aquatic turtle species, may be affected by the relative proportions of egg components, rather than simply its size, as Roosenburg and Dennis (2005) found with Diamondback Terrapin, *Malaclemys terrapin* eggs.

Lipid and protein content of *P. hilarii* egg yolks are similar to those reported for other pleurodiran species such as Murray Short-necked Turtle, *Emydura macquarii*, (Thompson et al. 1999) and Broad-shelled River Turtle, *Chelodina expansa*, (Booth 2003) and other cryptodiran turtles (Table 3). The relative similarity of the composition of lipids and proteins among certain members of the Chelidae family (Table 3) is reflected

in other ecological reproductive factors such as the development of different strategies at incubation and hatching time. *Phrynops hilarii* lays its eggs in early autumn, at which point the embryos immediately enter into a facultative diapause, hatching in the next spring-summer (Prieto 2015). Depending on the reproductive season, incubation for *P. hilarii* eggs may last up to one year due to the embryos being in a diapaused state over winter at a very early developmental stage, when the embryo is tiny and does not require much energy (Prieto 2015).

Strategies of developmental arrest in oviparous species that do not provide parental care after oviposition are essential for reproduction. Females provide the developing embryo with mechanisms to respond to environmental variation during its development outside the maternal oviduct. Proteins and lipids contained in the yolk and albumin provide the embryo with a primary source of food, water, and energy (Palmer and Guillette 1991). Changes in temperature associated with the surrounding environment can affect the water potential, slowing down metabolism and the rate of

TABLE 3. Dry lipid, protein, and mineral egg composition of freshwater turtle species. Values are expressed on 100 g dry of egg and egg yolk (n = 11). Data from: ¹This study, ²Thompson et al. (1999), ³Booth (2003), ⁴Whilhoft (1986), ⁵Rowe et al. (1995), ⁶Nagle et al. (1998), and ⁷Tunsaringkarnet al. (2013).

Species	Lipids (g)	Proteins (g)	Ca (mg)	Mg (mg)	K (mg)	Fe (mg)	Zn (mg)
Pleurodira							
Hilaire's Toadhead Turtle, <i>Phrynops hilarii</i> ¹	24.9	56.7	532.0	127.1	626.8	6.4	8.4
Murray Short-necked Turtle, <i>Emydura macquarii</i> ²	31.4	62.0	-	-	-	-	-
Murray River Turtle, <i>Emydura signata</i> ³	25.0	60.4	-	-	-	-	-
Broad-shelled River Turtle, <i>Chelodina expansa</i> ³	31.1	54.0	-	-	-	-	-
Cryptodira							
Snapping Turtle, <i>Chelydra serpentina</i> ⁴	33.8	54.9	-	-	-	-	-
Painted Turtle, <i>Chrysemys picta</i> ⁵	29.8	-	-	-	-	-	-
Striped Mud Turtle, <i>Kinosternon bauri</i> ⁶	34.1	-	-	-	-	-	-
Eastern Mud Turtle, <i>Kinosternon subrubrum</i> ⁶	32.9	-	-	-	-	-	-
Loggerhead Musk Turtle, <i>Sternotherus minor</i> ⁶	30.9	-	-	-	-	-	-
Eastern Musk Turtle, <i>Sternotherus odoratus</i> ⁶	28.2	-	-	-	-	-	-
Snail-eating Turtle, <i>Malayemys macrocephala</i> ⁷	31.6	45.9	440.0	70.0	430.0	-	-

oxygen consumption by the embryo, which would affect the rate of embryonic development (Ackerman 1994, Booth 2000). Higher temperatures favor greater evaporation of water and an increase in the rate of oxygen consumption, so that eggs deposited in hotter seasons or areas will develop faster than those deposited in season or areas with lower temperatures.

Chelodina expansa also nests predominantly during the autumn and winter and, as a response to low incubation temperatures embryos also enter a facultative diapause, enabling them to survive the cold season (Booth 2000, 2002; Bower and Hodges 2014). On the other hand, after hatching, Painted Turtle (*Chrysemys picta*) neonates remain in the nest throughout the winter and also require sufficient energy reserves to maintain a relatively large mass of tissue throughout the cold season (Rowe et al. 1995). *Emydura macquarii* eggs, however, exhibit similar yolk reserves to *C. picta*, *C. expansa* and *P. hilarii*, and the neonates emerge in late summer or early autumn, avoiding a prolonged incubation period. In this particular case, Thompson et al. (1999) argued that *E. macquarii* hatchling habitats are nutrient poor and therefore neonates require a large residual yolk rich in protein and lipid reserves at hatching. Snapping turtle *Chelydra serpentina* hatchlings, with lipid rations similar to *P. hilarii*, also emerge from their nests in autumn and begin to feed independently before winter. The variation present suggests that the composition of lipids and proteins in turtle eggs is not dependent on the nesting season, timing of neonate hatching, or phylogeny. Despite the proportional variability of lipids and proteins found among the eggs of chelonian species, both nutrients together are the principle energy source for developing reptile embryos (Noble 1991) and are generally found in the proportion of two to one, proteins to lipids (Thompson and Speake 2003). Studies regarding the mineral composition of turtle eggs are equally scarce. Mineral compositions (Ca, Mg, and K) recorded from *P. hilarii* eggs are within the range reported for Olive Ridley Turtle (*Lepidochelys olivacea*) eggs (Sahoo et al. 2010) and Snail-eating Turtle (*Malayemys macrocephala*) egg yolks (Tunsaringkarn et al. 2012, 2013; Table 3).

The egg components of *P. hilarii* did not vary with egg size (although the range of egg size was small in our study) as is generally assumed in studies of the reproductive ecology of oviparous species (Roosenburg and Dennis 2005), though Booth (2003) did find similar results in Murray River Turtle (*Emydura signata*) eggs. Usually, an increase in egg size corresponds with an increase in relative yolk size (Congdon et al. 1983; Wilhoft 1986; Finkler and Clausen 1997), though albumen accounts for most of the egg size variation in many bird and turtle species (Sotherland et al. 1990; Wallace et al. 2006). Egg quality needs to be taken into

account when egg-clutch size trade-offs are included in reproductive optimality models (Booth 2003). These models analyze energy allocation and are applied to natural populations using egg mass (easily measured) as a substitute for egg energy (more difficult to measure), under the assumption that egg energy density is uniform across all egg sizes (Smith and Fretwell 1974; Jaeckle 1995). This assumption, for example, would lead to the underestimation of energy allocation in clutches with large egg sizes if egg yolk size is relatively larger in larger eggs (Booth 2003).

While no significant correlations were detected in relation to egg components and the size of the egg, we did identify several correlations between egg yolk components. The yolk is the main depository for a wide range of ions and proteins (Simkiss 1991). The significant positive correlations found between the total protein and ions may be influenced by several agents, including that potassium, calcium, magnesium, and zinc are involved as cofactors in various enzymatic metabolic processes during embryonic development (Kaim and Schwederski 1994; Noronha and Matuschak 2002; Diaz García and Álvarez González 2009). The presence of Zinc at the membrane level affects a diverse array of receptors including ion channels K^+ , Na^+ and Ca^{+2} (Diaz García and Alvarez Gonzalez 2009), and in this study we found a positive and significant correlation between zinc and calcium. The correlation between zinc and potassium was positive but not significant.

This study is the first to analyze egg components of chelid turtle from South America, other than hormones (Paez et al. 2015), such as nutrients. It is an important first step in understanding the roles of nutrients in embryonic development and in analyzing developmental constraints in the evolution of maternal investment patterns in *Phrynops hilarii*. Further similar studies are necessary to better understand and compare the reproductive biology of other South American freshwater turtle species.

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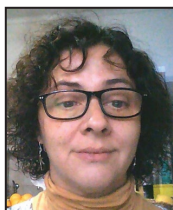
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