GROWTH AND ENERGETICS OF EMBRYOS OF THE GECKO, PHYLLODACTYLUS MARMORATUS, A SPECIES WITH HARD-SHELLED EGGS

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We measured water contents, growth of embryos and metabolic rates in hard-shelled eggs of the Australian gecko, *Phyllodactylus marmoratus*, throughout incubation to make comparisons between (1) the proportional water content at oviposition of eggs of *P. marmoratus* and flexible-shelled eggs of lizards; and (2) the dry-mass specific energy consumption during development in *P. marmoratus* and lizards with flexible-shelled eggs. Egg contents (i.e. excluding eggshell) contained nearly 80% water, higher than reported for any other squamate reptile. Eggs were laid at embryonic stages 26/27-29, which is slightly earlier than for most other lizards. Incubation lasted 79-84 days at 25 °C and net water loss averaged just under 3 mg. Metabolism reflected the size of embryos, with little growth and low rates of oxygen consumption during the first third of incubation. Thereafter, growth and oxygen consumption increased, with oxygen consumption slowing after day 70. This pattern is similar to that of other species of lizard. Water content of embryos fell from above 90% early in incubation to around 70% at hatching. Thus, the embryonic metabolic scaling factor was different when based on embryonic wet and dry mass. The dry-mass specific energetic cost of development in *P. marmoratus* was lower than other lizards, but this result was not related to having a hard-shelled egg. The respiratory exchange ratio suggests that embryonic metabolism is based on mixed protein and lipid, a pattern similar to that in flexible-shelled eggs of lizards, but different from birds.

Key words: *Phyllodactylus*, gecko, embryonic development.

INTRODUCTION

Geckos of the subfamily Gekkoninae are unusual among lizards because they lay eggs with hard calcareous shells (Packard, Tracy & Roth, 1977). Hard-shelled eggs of geckos have low conductances to gases (Dunson & Bramham, 1981; Dunson, 1982), which enables them to incubate in dry environments, such as under the bark of trees or in crevices among rocks, without desiccating. The unusual eggshell and "nest" environment suggest that other aspects of their development may be unusual, also.

The flexible-shelled eggs of most lizards absorb water during development (Packard, Packard & Boardman, 1982; Packard, Packard, Miller, Jones & Gutzke, 1985) and it is possible that many species must absorb water for proper development (Vleck, 1991; Ji, 1992). In contrast, water uptake by hard-shelled eggs of reptiles is minimal, if it occurs at all (Packard *et al.*, 1977). One would expect, therefore, that the water content of hard-shelled eggs might be higher than that of flexible-shelled eggs at the time of oviposition because the hard-shelled eggs are unable to supplement their water content during incubation.

The marbled gecko, *Phyllodactylus marmoratus*, is an Australian species of gekkonine lizard that lays hard-shelled eggs. It occurs in the "wetter parts of southern Australia" (Cogger, 1992). The reproductive

biology of *P. marmoratus* is well known (King, 1977; Doughty & Thompson, 1998), with mating occurring in autumn and females storing sperm over winter. Ovulation and fertilization using stored sperm occur in spring. This reproductive strategy makes *P. marmoratus* an ideal subject for studies of eggs, because females can be collected in spring and maintained in the laboratory in the absence of males until oviposition occurs.

We quantified the water content of fresh eggs, measured changes in embryonic water contents, and described embryonic growth in hard-shelled eggs of *P. marmoratus* to compare available data for species with flexible-shelled eggs. In addition, we measured embryonic metabolism (rates of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) throughout development to compare metabolic ontogeny in *P. marmoratus* with species of lizards with flexible-shelled eggs. These measurements allowed us to estimate total energy expenditure during development and to identify the energy substrate that fuels embryonic development. In particular, we compared the mass-specific energy consumed during development of embryonic *P. marmoratus* and lizards with flexible-shelled eggs.

MATERIALS AND METHODS

Gravid female geckos were collected in November, 1993, from beneath the bark of River Red Gum trees, *Eucalyptus camaldulensis*, along the banks of the River Murray in South Australia, between Murray Bridge and Blanchetown. Lizards were transported to the Univer-

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sity of Adelaide on the day of capture and housed individually in containers of 130 mm x 220 mm x 75 mm high in a room at 20-25°C, as described by Doughty & Thompson (1998). A window in the room ensured a natural light cycle and each container was heated from beneath at one end with electric heating cable to 32°C for 12 hours of the light phase.

Containers were inspected every morning for animal maintenance and to check for eggs, which were immediately removed from the cage without rotation. A unique number was written on each egg with a 3B graphite pencil and the egg was weighed on a mg balance. Eggs were placed in a box containing moistened Terra-lite grade 3 vermiculite with a water potential of -150 kPa, as determined using thermocouple psychrometry with a Wescor HR-33T microvoltmeter and C52 sample chamber. Eggs were placed on the surface of the vermiculite with 13-20 eggs per box. Each incubation box had a closely fitting lid and was incubated at 25±0.2°C. Individual eggs and the incubation box were weighed weekly and any mass loss from the box, assumed to be due to water loss (Packard, Packard, Miller & Boardman, 1988), was compensated for by the addition of distilled water. Incubation boxes were moved within the incubator daily to control for any undetected temperature variation within the incubator.

Six eggs were weighed and frozen whole on the day of laying (day zero) and subsequently lyophilized to give water content of whole eggs. Ten other eggs were dissected on the day of oviposition and the embryos were separated from the contents and the shells. Fresh whole wet egg mass and separate dry masses of eggshell and contents were determined for these eggs. Twenty-nine eggs, covering most embryonic stages, were dissected for embryonic staging between day 3 and day 75, after having their rate of metabolism measured (see below). A further nine eggs were dissected during incubation without metabolic rate being measured. Embryos were staged according to the scheme of Dufaure and Hubert (Porter, 1972), weighed to 0.01 mg and stored frozen until lyophilized in a Dynavac Freeze Drier Model FD-5. Water content was then calculated by subtraction. Wet and dry mass determination of yolk-free embryos and hatchlings exclusive of extra-embryonic fluid allowed calculation of water content of embryonic tissues.

In addition to the eggs that were dissected throughout incubation, we measured the rates of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) approximately twice per week in 14 eggs throughout incubation, using closed system respirometry as described by Thompson & Russell (1998). Eggs were sealed into glass jars of known volume at 25°C. Gas samples from the jars were analysed using an Ametek S-3A/II oxygen analyser and Ametek CD-3A carbon dioxide analyser. Output from the analysers was recorded on an IBM compatible PC computer using Datacan (Sable System Software). Barometric pressure was measured with a Compensiert
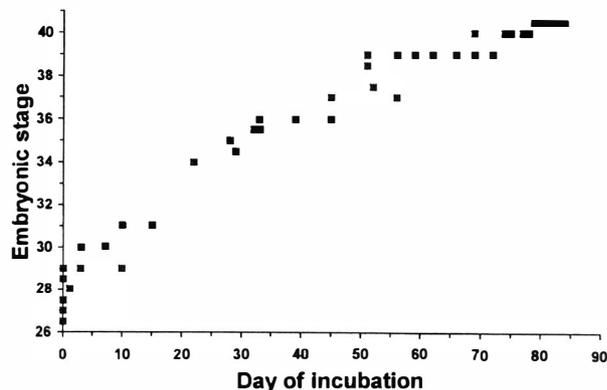

FIG. 1. Embryonic stage measured against time of incubation for *Phyllodactylus marmoratus* at 25 °C. Stages are based on tables of Dufaure and Hubert (Porter, 1972). Intermediate stages are arbitrarily assigned a 0.5 score (i.e. stage 38/39 is scored as 38.5) and hatchlings are scored as stage 40.5.

barometer and all values of $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ converted to STPD. Volume of gas in the respirometry chambers was adjusted for the volume of the egg by assuming an egg density of 1 (Douglas, 1990). Respiratory exchange ratio (RE) was calculated by dividing $\dot{V}_{CO_2}$ by $\dot{V}_{O_2}$. Total energetic cost of development was estimated by plotting $\dot{V}_{O_2}$ for individual eggs against incubation period, joining the points and calculating the area of the enclosed polygon. A mean value was then calculated from these individual estimates.

On the day of hatching, embryos were killed by cervical dislocation. Wet mass was measured to 0.01 mg, the hatchlings were dissected and internal yolk removed and weighed separately. All samples were stored frozen until lyophilized.

Means are presented ±1 SE and comparisons between means made using *t*-test. Linear regressions were done using the method of least squares and statistical significance was assumed if $P < 0.05$.

RESULTS

Phyllodactylus marmoratus layed one (26%) or two (74%) eggs. Incubation period at 25°C was 79-84 d (mean = 81.4±0.3 d, mode = 81 d, $n = 18$). Eggs were laid at embryonic stage 26/27-29 with most (seven of ten) being at stage 27 or 27/28. Most differentiation oc-
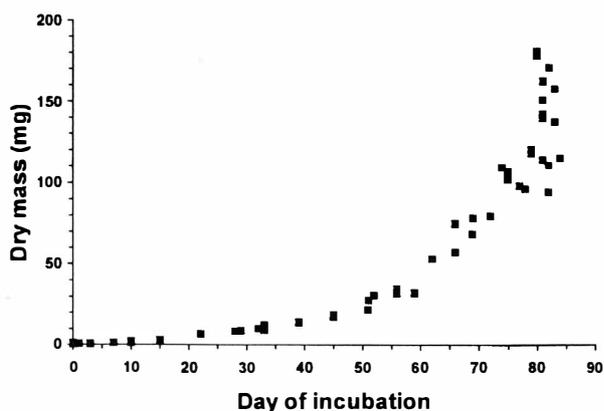

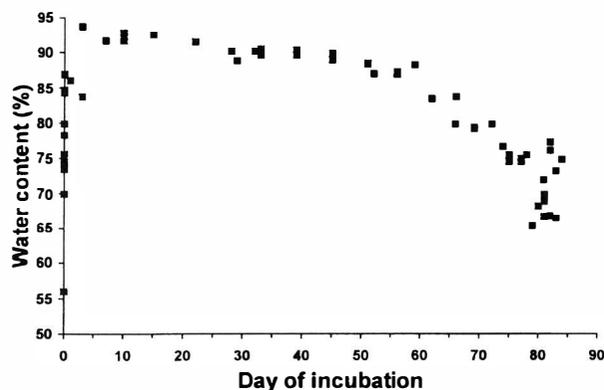FIG. 2. Dry mass of embryos and yolk-free hatchlings of *Phyllodactylus marmoratus* during incubation.

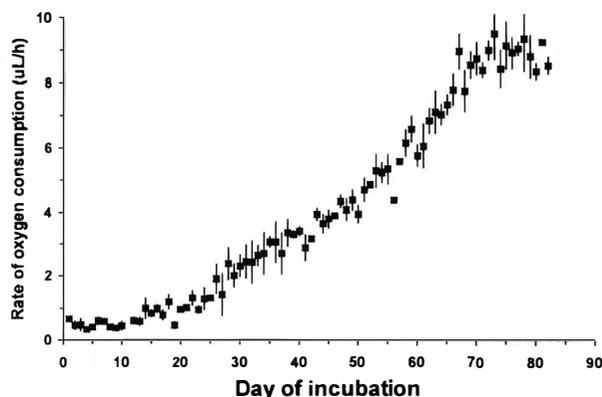FIG. 3. Water content of embryos of *Phyllodactylus marmoratus* throughout incubation.

FIG. 4. Mean metabolic rates of eggs of *Phyllodactylus marmoratus* oviposited in the laboratory throughout incubation. Bars represent 1 SE.

curred during the first 50 days and most embryos had reached the penultimate embryonic stage (Stage 39) by day 50 (Fig. 1). Embryonic growth was very slow until about day 50, after which growth accelerated rapidly and continued to increase until hatching (Fig. 2). Water content varied among day 0 embryos (Fig. 3). Water content of fresh whole eggs was 65.7±1.6% ($n=6$) and of the egg contents (i.e. egg minus the eggshell) was 77.9±0.9% ($n=10$). Initial mass of eggs killed on day 0 was not significantly different from initial mass of eggs allowed to hatch ($t = 0.43$, $P>0.05$). Water content of embryos was above 90% (Fig. 3) from day 3 to about day 50, after which it fell to 70.6±1.0% ($n = 14$; range = 65.4 - 77.4%) at hatching. The decrease in water content (Fig. 3) mirrors the increase in embryo size (Fig. 2). Egg mass remained almost constant during incubation. Mean initial egg mass was 628 ±8 mg (Table 1) for all eggs available and mean change in egg mass was a fall of 2.7±0.5 mg (range = -6 - +1 mg, $n = 18$) for all eggs that hatched. Yolk-free wet mass of hatchlings was 473±17 mg and yolk-free dry mass was 137±7 mg (Table 1).

Metabolic rates (Fig. 4) reflected embryonic growth (Fig. 2). Rates rose slowly from about 0.5 µLh⁻¹ to about 1.0 µLh⁻¹ for the first 25 days (or 30% of incubation). Thereafter, metabolic rate increased rapidly to reach a plateau of 8-10 µL.h⁻¹ between day 70 and hatching (Fig. 4). Integration of metabolic curves gave an estimate of 78.1±1.5 ml of O_2, equivalent to 1.51

±0.03 kJ of energy (Table 1). Thus, the dry mass-specific energy consumption during development was 11.3 ±0.5 kJg⁻¹ (Table 1).

A linear regression for RE plotted against day of incubation had a slope not significantly different from zero ($F = 0.29$, 1 df, $P = 0.592$) and an intercept of 0.77. For interpretation of our value of R, we have assumed that all metabolism is aerobic, that the only metabolic substrates are lipid and protein, and that the nitrogenous waste is stored as urea (Thompson & Russell, 1998). Based on the energetic equivalent of oxygen for different respiratory substrates (Schmidt-Nielsen, 1990), we estimate from an RE of 0.77 that embryonic energy metabolism in *P. marmoratus* is derived 40% from lipid and 60% from protein and has an energy equivalent of 19.163 kJL⁻¹ of O_2.

DISCUSSION

EMBRYONIC STAGE, GROWTH AND DIFFERENTIATION

Embryos of *P. marmoratus* are at a slightly earlier stage of development (about stage 27) than is typical for eggs of lizards (Shine, 1983). The reason for the difference between *P. marmoratus* and other species is not known, but may be common to geckos with hard-shelled eggs. Although the embryonic stage at oviposition was not actually reported in hard-shelled eggs of *Gekko japonicus*, it is very likely that, like *P. marmoratus*, they are at a relatively early stage of development because rates of oxygen consumption are too low to be detected when the eggs are first laid (Ji, 1992).

Differentiation of embryos is rapid after oviposition. Embryos are at the stage (29-31) normally associated with oviposition in lizards by day 5-10, although there is clearly some variation presumably associated with variation in stage at oviposition (Fig. 1). Differentiation continues rapidly until stage 36 at about 35 days. Thereafter, differentiation proceeds more slowly, with embryos spending longer at each stage, a pattern that is common among lizards (Thompson & Stewart, 1997).

In contrast to differentiation, growth of embryos is slow for the first half or more of incubation, after which

TABLE 1. Total oxygen consumption, its energy equivalent and energy consumed per g (mass-specific cost) of dry mass by eggs of *Phyllodactylus marmoratus*. Means are given ±1SE.

	Mean±SE	N	Range
Wet egg mass (mg)	628±8	74	499-774
Hatchling wet mass (mg)	473±17	14	356-571
Hatchling dry mass (mg)	137±7	14	95-181
Total VO_2 (ml)	78.7±1.53	14	67.35-87.87
Energy equivalent (J)	1508±29	14	1292-1684
Mass-specific cost (kJg⁻¹)	11.3±0.5	14	8.8-15.2

it accelerates (Fig. 2). This pattern in *P. marmoratus* is almost identical to that for *I. iguana* (Ricklefs & Cullen, 1973), a comparison that is particularly relevant given the similarities of incubation periods in *I. iguana* and *P. marmoratus*. The general pattern for these species is similar to that of altricial birds (Vleck & Hoyt, 1991), which is surprising given that lizards are essentially precocial. Embryonic growth of a dragon lizard, *Pogona barbata* (as *Amphibolurus barbatus*) (Packard *et al.*, 1985) shows broad similarity to growth of *I. iguana* and *P. marmoratus*, although there may be a slowing of growth towards the end of incubation to give a logistic growth curve, rather than the exponential curve of *I. iguana* (Vleck & Hoyt, 1991). Growth in the skink *Eumeces fasciatus* slows even more than in *P. barbata* late in development (Thompson & Stewart, 1997) to give a pattern similar to that of precocial birds. Calculation of individual growth rates for *P. marmoratus*, using the methods described by Hoyt (1987), shows that there is a decline in growth rate after a peak of 4.2 mg dry mass/day on day 71, although this decline is obscured by variation in sizes of embryos in Fig. 2.

The time of most rapid growth (Fig. 2) coincides with the time of slowest differentiation in *P. marmoratus* and *E. fasciatus* (Thompson & Stewart, 1997; Fig. 1) and with the most rapid loss of percentage water content of embryos in *P. marmoratus* and *I. iguana* (Ricklefs & Cullen, 1973; Fig. 3). Presumably, this is the time of ossification of bone and deposition of fat from reserves in the yolk, both tissues with low water contents.

WATER CONTENT AND MASS CHANGES DURING DEVELOPMENT

Eggs of *P. marmoratus* change in mass very little during development, with a mean net loss of water of only 2.7±0.5 mg. Flexible-shelled eggs incubated at a water potential similar to that used for *P. marmoratus* would gain water for the first half or more of incubation and then lose some prior to hatching, resulting in a net uptake of water during incubation (Packard *et al.*, 1985). No gain of mass was measured at any time during development. The small loss of mass in *P. marmoratus* presumably reflects the low shell conductance of hard-shelled eggs of geckos (Dunson & Bramham, 1981; Dunson, 1982) and is probably typical of hard-shelled eggs of other gekkonine lizards. The metabolism of both lipid and protein results in the production of water (Withers, 1992). Since there was a small net loss of water during development, all the metabolic water generated during development must also have been lost.

Although the water contents of eggs of many species of birds have been reported as a percentage of mass of egg contents, excluding the shell, it is not clear that data for most reptiles do not include the shell (Vleck, 1991).

Knowledge of the mass and water content of eggs independent of the shell is important in reptiles because some species, such as *P. marmoratus*, with a calcareous shell have a greater relative shell mass than others with flexible-shelled eggs that lack extensive deposits of calcium. There are, however, some data for water content of egg contents for flexible-shelled eggs for comparison with eggs of *P. marmoratus*. The mean water content of fresh eggs (excluding the shell) for four species of squamates (the agamid lizard, *Pogona barbata*, the snake *Coluber constrictor* (Packard *et al.*, 1985) and the skinks *Eumeces fasciatus* (Thompson & Stewart, 1997), *Menetia greyii* (Thompson & Russell, 1998)) is 64.5±3.9% (range: 59.0 - 75.9%). Only one of those is above 70% (*M. greyii*) and all contain a smaller proportion of water than eggs of *P. marmoratus*. Thus, eggs of *P. marmoratus* contain relatively more water than other species so far studied, but comparative data are few. Eggs of another species with hard-shelled eggs, *Gekko japonicus*, are reported to be 74% water, but the report does not make clear whether the measurement includes the eggshell (Ji, 1992). If the value represents shell-free egg contents, then the water content of *G. japonicus* and *P. marmoratus* are very similar; if the value includes the shell, then eggs of *G. japonicus* would contain proportionally more water than eggs of *P. marmoratus*, strengthening the suggestion that hard-shelled eggs have high relative water contents at the time of oviposition. The water content of flexible-shelled eggs of squamates is much lower than that of altricial (mean = 84.3% water) and precocial (mean = 74.7% water) birds (Vleck, 1991), whereas the water content of eggs of *P. marmoratus* is similar to, and well within the range of values for precocial birds (Vleck & Hoyt, 1991). Since water uptake does not occur in eggs of *P. marmoratus* during development, and since neonates of *P. marmoratus* are precocial in an avian sense, this similarity is not unexpected.

The variation in estimates of water content of embryos on day zero reflects the very small mass (Fig. 2) and fragility of early embryos. Thus, small errors in measurements from inadvertent inclusion of small amounts of yolk may result in proportionally large errors in the estimate of water content. Nevertheless, all estimates on day zero are lower than those from days 3 - 50, so it is likely that early embryos rapidly take up water. Embryos of *Iguana iguana* also take up water during the first few days of incubation (Ricklefs & Cullen, 1973). Thereafter, although embryonic *I. iguana* generally contain relatively more water than *P. marmoratus*, they show a similar decline in relative water content (Ricklefs & Cullen, 1973).

METABOLIC RATES

Because of the large change in percent-age water content of embryos during development, we plotted regressions of rates of oxygen consumption against wet

and dry mass for all embryos and hatchlings that were killed immediately after measurements ($n = 35$). The resulting regressions are:

$$\ln \dot{V}_{O_2} = 0.18 + 0.69 \ln \text{ wet mass}$$
$$(r^2 = 0.89, F = 273.39, 1 \text{ df}, P < 0.001)$$

$$\ln \dot{V}_{O_2} = 2.30 + 0.45 \ln \text{ dry mass}$$
$$(r^2 = 0.88, F = 235.05, 1 \text{ df}, P < 0.001)$$

where $\dot{V}_{O_2}$ is in $\mu L h^{-1}$ and dry mass is in mg. A result of the changes in water content of embryos during development is that the metabolic scaling factor for $\dot{V}_{O_2}$ is different for wet embryos (0.69) and dry embryos (0.45). Both scaling factors are lower than the 0.76 for dry mass of the scincid lizard *Eumeces fasciatus* (Thompson & Stewart, 1997). The relevance of the different scaling factors in *P. marmoratus* and *E. fasciatus* is not known.

The shape of the curve that describes the increase in $\dot{V}_{O_2}$ during incubation is similar to that for other species of lizards (e.g. Thompson & Stewart, 1997) with little rise for the first part of incubation (about 14 days in *P. marmoratus*), followed by a rapid rise until late in development when the rate of increase slows to reach a plateau. There appears to be no decline in $\dot{V}_{O_2}$ late in incubation (Fig. 4) as there is in some species of reptiles (Thompson, 1989). $\dot{V}_{O_2}$ levels off at about the same time (day 71, Fig. 4) that growth rate declines, a pattern typical of precocial birds (Hoyt, 1987) and some other lizards (Thompson & Stewart, 1997).

The dry mass-specific energy consumption during development in *P. marmoratus* is lower (11.3 ± 0.5 kJg^1) than shown in any other species of lizard (range: 12.4 kJ.g^{-1} for *Eumeces fasciatus* to 19.6 kJg^{-1} for *Morethia adelaidensis*) (Ji, 1992; Thompson & Russell, 1999; Thompson & Stewart, 1997; Thompson, Speake, Russell & McCartney, 1998a; Vleck & Hoyt, 1991) but is within the range (9.7-21.9 kJg^{-1}) for birds (Booth & Thompson, 1991). This result, however, is probably not associated with the hard eggshell, because the dry mass-specific cost of development in another gecko with hard-shelled eggs, *Gekko japonicus*, is 15.2 kJg^{-1} (Ji, 1992), close to the mean of 15.6 1.1 kJg^{-1} reported for lizards (Thompson & Russell, 1999).

RESPIRATORY EXCHANGE RATIO AND METABOLIC SUBSTRATE

An important result from the metabolic measurements is that RE is above 0.71, confirming that mixed protein and lipid is used as a metabolic substrate during incubation. Similar values of RE have been reported in other lizards (Thompson & Russell; 1999; Thompson & Stewart, 1997). Considering the relatively lower energy density of protein compared to lipid (Schmidt-Nielsen, 1990) and the small importance of protein as a metabolic substrate for embryonic birds

and turtles (Romanoff, 1967; Rahn & Ar, 1974; Thompson, Speake, Russell, McCartney, 1998b), it is puzzling why lizards rely so heavily on metabolism of protein to fuel embryonic development. Greater reliance on lipids as a metabolic fuel would enable a smaller egg of equivalent energy density to be produced. It appears, however, that female *P. marmoratus* are able to accommodate a range of egg sizes (Doughty & Thompson, 1998). The striking similarity of the proportion of lipids and protein used as metabolic substrates during development in *P. marmoratus* and species with flexible-shelled eggs (Vleck & Hoyt, 1991; Thompson & Stewart, 1997; Thompson & Russell, 1999) suggests that the utilization of a mixed metabolic substrate during development may be general in lizards.

CONCLUSION

This study has shown that the hard-shelled eggs of *P. marmoratus* share aspects of embryonic growth, metabolism and metabolic substrates with other lizards. The main differences thought to be associated with having a hard eggshell are the initial water content and loss of water during incubation. The utilization of protein as a major metabolic substrate during development is similar to other species. Further comparative study of the relationships of metabolism of protein, metabolic water production and net water exchanges with the environment in both hard-shelled and flexible-shelled eggs during incubation is required to understand the basis of the difference in the relative use of lipids and proteins as energy substrates in embryos of lizards, compared to turtles (Thompson *et al.*, 1998b) and birds (Rahn & Ar, 1974).

ACKNOWLEDGMENTS

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