

Does Low Gas Permeability of Rigid-Shelled Gekkotan Eggs Affect Embryonic Development?

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ABSTRACT

Parchment-shelled eggs are characteristic of most squamates, including the basal clades of gekkotan lizards. The majority of gekkotan lizards, however, produce rigid-shelled eggs that are highly impermeable to gas exchange; eggs are laid in dry sites and experience a net loss of water during incubation. We tested the hypothesis that the 1,000-fold lower rate of oxygen diffusion through the shells of rigid- compared to parchment-shelled eggs imposes a physiological cost on development. To do this, we contrasted species with rigid and with parchment shells with regards to (1) rates of embryonic metabolism and (2) rates and patterns of development of the yolk sac and chorioallantois, the vascularized extra-embryonic membranes that transport oxygen to embryonic tissues. Metabolic rates of embryos from the rigid-shelled eggs of *Gehyra variegata* did not differ from those of the parchment-shelled eggs of *Oedura lesueurii*. Moreover, maximum metabolic rates of gekkotans with rigid shells did not differ from those of gekkotan or scincid lizards with parchment shells. In contrast, the yolk sac covered more of the surface area of the egg at oviposition, and the chorioallantois reached its full extent earlier for the species with rigid shelled eggs (*Chondrodactylus turneri*, *G. variegata*) than for the species with parchment-shelled eggs (*Eublepharis macularius*, *O. lesueurii*). Differences in the temporal patterns of yolk sac and chorioallantois development would thus serve to compensate for low rates of oxygen diffusion through rigid shells of gekkotans. *J. Exp. Zool.* 319A:259–267, 2013. © 2013 Wiley Periodicals, Inc.

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Most squamate reptiles (lizards and snakes) lay parchment-shelled eggs that are highly permeable both to water (vapor and liquid) and to respiratory gases (Deeming and Thompson, '91). The water dynamics of these eggs are complexly related to the physical environment of the egg and to factors intrinsic to the egg and the developing embryo (Ackerman et al., '85; Adams et al., 2010). Successful hatching, however, depends on a net water uptake by eggs (Thompson and Speake, 2004). Parchment-shelled eggs must therefore incubate within substrates where the atmosphere is near saturated so that water loss is less than water uptake. Diffusive water loss, however, is usually not a problem for parchment-shelled eggs because typical nesting environments in soil have very high relative humidities (RH > 99%, Tracy et al., '78) that

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favor net water uptake. The availability of oxygen in nests is also not usually limited during development because eggs of most squamates are small (several grams or less) and nests are located within several centimeters of the surface where diffusion is sufficient to replace oxygen used in metabolism (Packard and Packard, '88; Booth, '98). In general, water and oxygen are unlikely to limit embryonic development in the parchment-shelled eggs of squamates. This conclusion does not apply, however, to those squamates that produce rigid-shelled eggs.

Rigid-shelled eggs characterize the monophyletic clade comprised of sphaerodactylid, gekkonid, and phyllodactylid geckos (Gamble et al., 2012). Shells are heavily mineralized and exhibit highly reduced gas permeability compared to those of parchment-shelled eggs (Deeming and Thompson, '91). These gecko eggs are morphologically and functionally comparable to those of birds (Packard and Hirsch, '89; Packard and DeMarco, '91; Andrews, 2012). Rigid-shelled gecko eggs contain all the water needed for embryonic development at oviposition, and egg mass decreases during incubation via the diffusion of water vapor through the shell (Deeming and Unwin, 2004; Andrews, 2012). For birds at least, the amount of diffusive water loss is regulated evolutionarily to be "just right" for normal development (Paganelli, '91). If water loss is too low in avian eggs, an air cell does not form or is not of sufficient size for chicks to ventilate their lungs prior to pipping (Ar, '91). On the other hand, if water loss is too high, the embryo will not have sufficient water to complete development, and to hatch successfully. Nests of gekkotans that lay rigid-shelled eggs are similar to those of birds in that the relative humidity of the air surrounding eggs is relatively low. Gecko eggs are often placed in aboveground cavities, glued to exposed surfaces of trees or rocks, or attached to overhanging rock faces (Dunson, '82; Henkel and Schmidt, '95; Deeming and Unwin, 2004; Ineich, 2010). While rigid-shelled eggs may also be buried in debris on the ground, the substrates in these nest sites are relatively dry during all or part of the incubation period (Brown and Duffy, '92; Köhler, 2005). Low water vapor permeability of the shell allows rigid-shelled gekkotan eggs to be laid in dry environments without danger of desiccation. On the other hand, low permeability to oxygen could make obtaining oxygen across the eggshell for embryonic development a problem.

Gekkotans that produce rigid-shelled eggs exhibit a suite of life history features distinct from those of gekkotans that produce parchment-shelled eggs (Pike et al., 2012). For example, species with rigid-shelled eggs generally have smaller adult body sizes, eggs, and hatchlings than those with parchment-shelled eggs. These differences are associated with the water economy of eggs and physical constraints on the size of rigid-shelled eggs relative to female body size (Kratochvil and Frynta, 2006). Gekkotans that lay rigid-shelled eggs also oviposit when embryos are at earlier stages of development and have longer incubation periods than gekkotans that lay parchment-shelled eggs (Pike et al., 2012). These observations suggest that the low permeability of the rigid

shell to oxygen could affect embryonic development. For example, embryos may be at early stages at oviposition because development was arrested while in the oviduct by low oxygen availability (Andrews and Mathies, 2000; Rafferty et al., 2013). Even after oviposition, oxygen availability may not be sufficient for optimal embryonic growth. Reduction in ambient PO_2 reduces embryonic metabolism and the rate of development in a graded fashion (Parker et al., 2004; Stahlschmidt and DeNardo, 2008). Thus, even small reductions in PO_2 below normal ambient conditions can have a negative impact on development.

Does the rigid shell of gecko eggs reduce oxygen diffusion sufficiently to affect development? This question can be addressed theoretically using Fick's law (Paganelli et al., '78; Booth, '98). If the premise that the rigid shell can limit oxygen availability to the embryo is correct, internal PO_2 of rigid-shelled eggs should be substantially lower than ambient while internal PO_2 of the highly permeable parchment-shelled eggs should be similar to ambient PO_2 . Oxygen conductances (GO_2) of rigid- and parchment-shelled eggs (0.75 and 828 $mL O_2 day^{-1} kPa^{-1}$, respectively) were estimated from mean water vapor conductances (0.73 and 802 $mg day^{-1} kPa^{-1}$, respectively, Andrews, 2012) for eggs with egg surface areas of 3.3 and 3.8 cm^2 for rigid-shelled eggs (spherical) and parchment-shelled eggs (oblong) that each produce a 0.4 g hatchling. $VO_2 max$ of an egg that produces a 0.4 g hatchling is 2.7 $mL O_2 day^{-1}$ at 26°C (mean of species listed in Table 1). Assuming 50% RH and 100% RH for nests of rigid- and parchment-shelled eggs, respectively, corresponding ambient PO_2 s would be 20.8 and 20.5 kPa. Resultant internal PO_2 s of rigid- and parchment-shelled eggs are 17.2 and 20.5 kPa from the equation, $PO_2 internal = PO_2 nest - VO_2 max / GO_2$. Given these calculations, low oxygen conductance of rigid-shelled eggs of gekkotans has the potential to negatively affect development.

We made two predictions to test the hypothesis that low oxygen conductance of the eggshell affects the development of gekkotan embryos in rigid-shelled eggs. The first prediction was that metabolic rates of embryos of geckos that produce rigid-shelled eggs are lower than those that produce parchment-shelled eggs, at least at high temperatures or/and late in development when oxygen demand is the greatest. To test this prediction, we measured VO_2 of embryos of a rigid-shelled species and a parchment-shelled species during the last half of development at four incubation temperatures. We also collected information from the literature on peak embryonic metabolism ($VO_2 max$) of gekkotan and scincid lizards for a broader comparative perspective.

The second prediction is that the extra-embryonic membranes responsible for transporting oxygen to the embryo will form earlier relative to embryonic stage in species that produce rigid-shelled eggs compared to those that produce parchment-shelled eggs. Oxygen is delivered to early stage embryos through simple diffusion from the oviduct or the atmosphere through the shell to embryonic tissues. Given the inefficiency of simple diffusion,

Table 1. VO_2 max and associated life history features of gekkotan and scincid embryos and hatchlings.

Family: Species	VO_2 max (mL/h)	Hmass (g)	Temp (°C)	IncPer (day)	Stage	Source
Gek: <i>Chondrodactylus turneri</i> (RS)	–	1.16	28	70	25	RMA
Gek: <i>Gehyra variegata</i> (RS)	0.059	0.42	25	105	26	This paper
Gek: <i>Gekko japonicus</i> (RS)	0.231	0.47	32	42.9	23.5	2
Phy: <i>Christinus marmoratus</i> (RS)	0.09 ^a	0.47	25	81.4	27.5	3
Dip: <i>Oedura lesueurii</i>	0.081	0.40	28	–	30	This paper
	–	0.40	25	58	30	
Eub: <i>Eublepharis macularius</i>	–	2.30	28	51	28	RMA
Pyg: <i>Aprasia inaurita</i>	0.076	0.32	25	74	27 ^b	1
Pyg: <i>Delma mollerii</i>	0.189	1.04	25	87	27 ^b	1
Sci: <i>Plestiodon anthracinus</i>	0.13	0.42	27	27	31 ^b	4
Sci: <i>Plestiodon fasciatus</i>	0.18	0.49	27	25	31	4
Sci: <i>Menetia greyii</i>	0.027	0.068	29	30	28	5
Sci: <i>Morethia boulengeri</i>	0.042	0.15	25	58	29.7	6
Sci: <i>Morethia adelaidensis</i>	0.055	0.20	25	58	29.1	6
Sci: <i>Lampropholis guichenoti</i>	0.056	0.16	25	40	28	7
Sci: <i>Bassiana duperreyi</i>	0.080	0.28	25	41	29.5	7

Hmass, hatchling mass, Temp, incubation temperature, IncPer, incubation period, and Stage, stage at oviposition. Gekkotan families: Dip, Diplodactylidae; Eub, Eublepharidae; Pyg, pygopodidae; Gek, Gekkonidae; Phy, Phyllodactylidae. Sci, Scincidae. RS, species with rigid shells, undesignated have parchment shells; RMA, Robin Andrews, unpublished data.

Sources: 1. M. B. Thompson, unpublished, 2. Wang and Ji ('97); X. Ji, pers. comm.; 3. Thompson and Russell ('99a); 4. Thompson and Stewart ('97); 5. Thompson and Russell ('98); 6. Thompson and Russell ('99b); 7. Booth et al. (2000).

^aCorrected value for VO_2 max; the value in Thompson and Russell ('99a) was low by a factor of 10.

^bStage at oviposition based on a congener or related species.

accelerated development of the yolk sac (YS) would enhance the delivery of oxygen to the embryo early in development when the YS is the only vascularized extra-embryonic membrane. Similarly, accelerated development of the CAM would enhance oxygen delivery to the embryo later in development when the CAM becomes the predominant source of oxygen for the embryo (Andrews, 2004). We therefore contrasted the rate and timing of growth of the YS and the CAM for two gekkotan species that produce rigid-shelled and two that produce parchment-shelled eggs.

MATERIALS AND METHODS

Our objective was to compare embryonic metabolism and extra-embryonic membrane development of gekkotan species that produce rigid-shelled (RS) eggs and that produce parchment-shelled (PS) eggs. We chose pairs of similarly sized species of rigid- and parchment-shelled species to study to reduce problems of scaling overall, hence, the relative large *Chondrodactylus turneri* (RS) and *Eublepharis macularius* (PS) and the relatively small *Gehyra variegata* (RS) and *Oedura lesueurii* (PS). We were not able to make identical observations on all species. (1) Because of unusually cold weather, *O. lesueurii* did not reproduce in 2011 and we unable to collect data on yolk sac development in parallel with that collected on *G. variegata*. (2) Metabolic data were not collected for *C. turneri* and *E. macularius* embryos and we

therefore used information for other gekkotan species from the literature for comparative analyses. (3) To make biologically relevant observations, incubation temperatures must be within the optimal temperature range for development. Higher and lower temperatures reduce performance, for example, kill embryos or cause developmental abnormalities (Andrews and Schwarzkopf, 2012). The optimal temperature range exhibits substantial interspecific variation, and in some cases, precludes comparisons at the same incubation temperature. For example, hatching success of *C. turneri* and *E. macularius* is very low at 25°C (Andrews, 2012, unpublished data) while 25°C falls within the range of natural nest temperatures of *O. lesueurii* and *G. variegata* (Bustard, '69; Pike et al., 2010). Incubation temperatures for the former pair in our study were thus higher than for the latter pair and comparative analyses of metabolism incorporated incubation temperature as a covariate to account for variation in temperature during observations of metabolism.

Source and Maintenance of Gekkotan Lizards and Eggs

Eggs of *C. turneri* and *E. macularius* were obtained during the period January–July 2011 from breeding colonies maintained in Andrews' laboratory at Virginia Tech ($n = 24$ and 32 , respectively). Eggs of *G. variegata* ($n = 20$) were collected in the field and obtained from 28 gravid females collected in December 2009 and

December 2011 at the Pilliga State Forest, New South Wales, Australia (30°48'S 148°59'E). Eggs of *O. lesueurii* were obtained from 26 gravid females collected in October 2009 at Royal National Park, New South Wales (34°07'S 151°04'E). Gravid females of the Australian species were maintained in Thompson's laboratory at the University of Sydney. Cages were checked daily for eggs. Eggs were weighed (mass, g) at oviposition or shortly after collection (*G. variegata*).

Eggs of *C. turneri* and *E. macularius* were incubated at 28.5°C and eggs of *O. lesueurii* and *G. variegata* were incubated at 25°C. During incubation, eggs were half buried in moistened cocopeat or vermiculite (at water potentials of approximately -200 kPa) (*E. macularius*, *O. lesueurii*) or placed on the surface of moistened vermiculite (*G. variegata*) or dry sand (*C. turneri*). Embryos were either sampled at oviposition or after designated periods of incubation. Incubators were checked at least daily and hatchlings were weighed within 24 hr of hatching.

Embryonic Metabolism

Eggs were incubated at 25°C except for the 16–19 hr periods when embryos were in respirometers. Metabolic rates of *G. variegata* embryos were measured during the period January 19–February 7, 2010 when embryos were 55–105 days old and of *O. lesueurii* eggs from December 29, 2009 to January 19, 2010 when embryos were 27–59 days old. Metabolism was thus measured during the latter half of the incubation period when the relationship between VO_2 and age is approximately linear (Thompson and Russell, '99a). Metabolic rates were measured at 27.7 ± 0.12 , 29.7 ± 0.10 , 31.5 ± 0.17 , and $33.5 \pm 0.05^\circ\text{C}$. The order of temperature treatments was randomized except that each sequence ended with 33.5°C because we did not know the upper limit of short-term temperature tolerance of either species. Metabolism of each egg was measured at several times during incubation.

The rate of oxygen consumption (VO_2) was measured using closed system respirometry (Thompson, '89; Thompson and Stewart, '97). Eggs were positioned in small aluminum tare pans along with a small piece of filter paper moistened with 0.1 mL of distilled water and placed in glass metabolic chambers (465–474 mL). Chambers were sealed and placed in an incubator set at one of the four pre-designated constant temperatures. After 16–19 hr, a gas sample was taken from each chamber into a 60 mL syringe via a three-way stopcock. Gas samples were injected into a continuous stream of air that was scrubbed of water vapor and carbon dioxide with a series of Drierite, Ascarite, and Drierite. The airstream was drawn through a single channel of a FC-2 Oxxilla II (Sable Systems) oxygen analyzer at approximately 22 mL min^{-1} . Output from the oxygen analyzer was recorded using a UI-2 data acquisition interface and ExpeData software (Sable Systems). The volume of each chamber was measured beforehand by filling it with water at a known temperature, weighing the water and calculating the volume for the density of water at that temperature. The volume of dry air in each chamber was then determined by subtracting the volume of the

egg, filter paper, tare pan, liquid water, and water vapor (assuming saturation), based on the weight and density of each of these objects, from the volume of each chamber. Barometric pressure and temperature in the metabolic chambers were measured at the time the chambers were sealed. Rates of oxygen consumption were calculated using equations of Vleck ('87) and corrected to STPD.

We also collected data on VO_2 max from the literature to make a broader comparison of the metabolism of geckos with that of other lizards. We limited the comparison to scincid lizards because they have the largest number of observations on embryonic metabolism of any family of lizards. This has the benefit of comparing embryonic metabolism between two of the most basal squamate lineages (Townsend et al., 2004). We also used estimates of VO_2 max because they are commonly reported, and thus broadly comparable across taxa.

Extra-Embryonic Membranes

Eggs were preserved in 10% formalin when they were sampled. The length and width of eggs were measured with a dial calipers. Shells were carefully removed so the extent of the vascularized YS and CAM could be seen on the surface of the egg or adhering to the inner surface of the shell. The amount of development of these membranes was scored as the % coverage of the surface area of the egg. This convention works well for the CAM because its area increases monotonically during development until the outer surface of the egg is entirely or largely covered. Development of the YS is more complicated (Stewart et al., 2012). At stage 30 or earlier, the area of the vascularized YS can be measured and quantified as a percentage of the surface area of the egg. As the embryo increases in size, however, the YS becomes increasingly associated with the abembryonic pole. We therefore evaluated the area of the YS from the perspective of the abembryonic pole, that is, 90% coverage was 100% minus the 10% of the area yet to be covered by the YS and did not attempt to correct for development events at the embryonic pole of the egg. When the area of the membranes were less than 15% of the surface area of the egg (diameters of 12 mm or less), the major and minor axes of the YS and CAM were measured with an ocular micrometer and areas calculated using the formula for the area of an ellipse. Surface area was expressed as a percentage of the surface area of the egg independently for both membranes. The surface area of the near spherical rigid-shelled eggs was estimated as the area of a prolate spheroid and the surface area of the elongate parchment-shelled eggs was estimated as the area of a cylinder with hemispherical caps. When the area covered by the membranes was larger than 15%, areas of the membranes were visually estimated by comparison with scale diagrams of ellipses representing membranes superimposed on outlines of eggs indicating coverage of 5%, 10%, 15%, 20%, 25%, 35%, 40%, and 50% that accounted for the curvature of the egg. After dissection, embryos and associated membranes were transferred to 70% ethanol for permanent storage.

The nominal embryonic stage was assigned based on Dufaure and Hubert's ('61) normal table of development. In this scheme, embryonic development starts at stage 1 with the initiation of cell division (cleavage) and ends at stage 40 when hatching/birth occurs. Stages 5–9 are associated with gastrulation, 10–26 with neurulation and organogenesis, and 27–31 with the formation of limb buds and their development into paddle-like structures. For the great majority of lizards, oviposition occurs during limb bud stages (Andrews and Mathies, 2000; Andrews, 2004).

Statistical Analyses

Preliminary analyses of metabolism revealed significant pair-wise interactions between the class variables temperature and species and between these class variables and relative age (day since oviposition divided by incubation period), the covariate. We therefore report results of ANCOVAs that compared VO_2 of the two species independently at each temperature and that used relative age as a covariate to adjust for variation in embryo age.

For comparisons of VO_2 max among species, gekkotans with rigid-shells were compared with parchment-shelled gekkotans plus skinks and gekkotans overall were compared with skinks using one-factor ANOVAs. Because both incubation temperature and size of embryos affect metabolism (Deeming and Thompson, '91), the data used in analyses were the studentized residuals of the multiple regression between VO_2 max (dependent variable) and incubation temperature and log 10 hatchling mass (independent variables).

Plots of the egg surface area covered by the YS and by the CAM as a function of stage resembled a logistic relationship, but with abrupt transitions between 0% and 100% coverage (see Andrews, 2007). To best represent the areas of these membranes as a function of embryonic stage, the data were truncated to exclude observations in the long horizontal "tails." Excluded observations for each species included those made prior to the initial development of these membranes (area = 0%) and those made after coverage was 95% or more with the exception of the first observation for which the surface area covered by these membranes area had reached 95%. The remaining observations were log 10 transformed for linearity with respect to stage. Membrane coverage among species was contrasted with one-factor ANCOVAs for which species was the main factor and stage was the covariate. As we predicted differences in slope between shell types, initial models included a species by stage interaction term.

Statistical analyses were conducted with JMP Software (Copyright 2007, SAS Institute, Cary, NC, USA). Means are given ± 1 standard error (SE).

Animal Protocols and Collecting Permits

The University of Sydney Animal Ethics Committee approved experimental protocols [numbers L04/10-99/3/3026 and L04/8-2009/2/5112] and lizards in Australia were collected under NSW

Scientific License no. S10693 to MBT. The Virginia Tech Institutional Animal Care and Use Committee approved the research protocol for RMA's studies at Virginia Tech on April 6, 2010 (IACUC No. 10-041-BIOL).

RESULTS

Embryonic Metabolism

The metabolic rate of *G. variegata* embryos increased with temperature overall while that of *O. lesueurii* embryos increased between 27.7 and 29.7°C but then plateaued (Fig. 1). Metabolic rates of *G. variegata* and *O. lesueurii* embryos differed at 29.7°C ($F_{1,18} = 23.6$, $P < 0.001$, $R^2 = 0.87$) but not at 27.7, 31.5, and 33.5°C (P s = 0.06, 0.81, and 0.35, respectively).

Peak metabolic rates of the three gecko species that lay rigid-shelled eggs, the three gecko species that lay parchment-shelled eggs, and seven skink species (Table 1) were similar. VO_2 max of rigid-shelled gekkos and parchment-shelled gekkos plus scincids did not differ ($F_{2,10} = 1.04$, $P = 0.39$) nor did the VO_2 max of gekkos overall versus scincids ($F_{1,11} = 2.28$, $P = 0.16$).

Extra-Embryonic Membranes

At oviposition, the area of the egg covered by the YS was greater for *C. turneri* and *G. variegata*, species with rigid shells, than for *E. macularius*, the species with a parchment shell. By stages 32–33, however, the yolk sac covered 100% of the egg of all species (Fig. 2). As expected, the interaction term in the preliminary ANCOVA was highly significant indicating heterogeneity in the

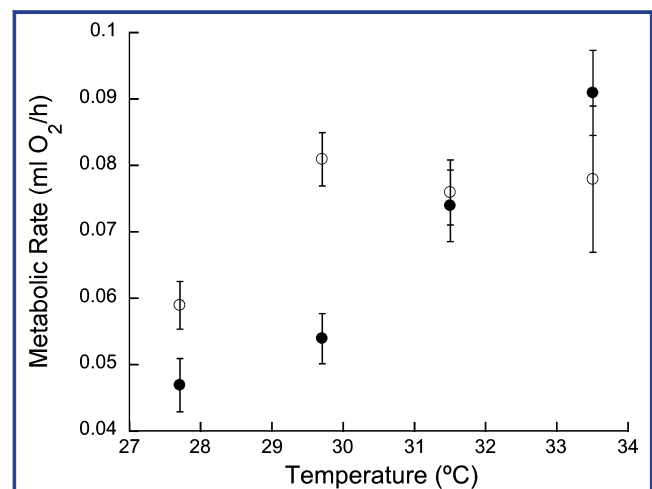


Figure 1. Metabolic rates of *G. variegata* (filled circles) and *O. lesueurii* (open circles) embryos as a function of temperature. These species have rigid- and parchment-shelled eggs, respectively. Values plotted are least squared means ± 1 SE of pair-wise comparisons.

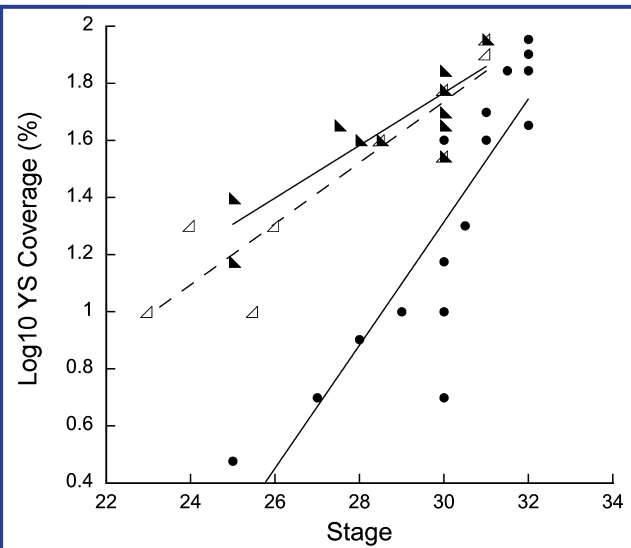


Figure 2. Log 10 coverage (%) of eggs by the yolk sac (YS) as a function of stage for gekkotans with rigid shells [triangles: *C. turneri* (closed triangles, solid regression line), *G. variegata* (open triangles, dashed regression line)] and for gekkotans with parchment shells [*E. macularius* (closed dots, solid regression line)].

increase in YS coverage as a function of embryonic stage (Table 2). The regression slopes for *C. turneri* and *G. variegata* ($F_{1,18} = 0.55$, $P = 0.37$, slopes test) and intercepts ($F_{1,19} = 0.93$, $P = 0.34$, ANCOVA) did not differ. We therefore concluded that the pattern of YS development differed between the species with rigid shells and the species with a parchment shell. For example, YS coverage for *C. turneri* and *G. variegata* reached 50% earlier in development (stages 29.2 and 29.7, respectively) than did YS coverage for *E. macularius* (stage 31.8; from individual species regressions).

Expansion of the CAM was initiated at about the same stage for both rigid- and parchment-shelled eggs; at stage 30, CAM coverage overall was 10–15% (1.0–1.2 log 10 units, Fig. 3). At any given stage somewhat later in development, however, the relative area of the CAM was higher and reached 100% coverage of the egg earlier for rigid- than the parchment-shelled eggs. As expected, the species by stage interaction term in an overall analysis was significant (Table 2). We therefore conducted pair-wise analyses to determine how the relationship between CAM coverage and stage differed for rigid- versus the parchment-shelled species (Table 2). Regression slopes and intercepts of *C. turneri* and *G. variegata*, the two rigid-shelled species, did not differ. The rigid-shelled *C. turneri* had higher regression slopes than both parchment-shelled species ($P_s < 0.001$, slopes tests, all comparisons). In contrast, while the rigid-shelled *G. variegata* had a higher regression slope than the parchment-shelled *O. lesueurii*, regression slopes of *G. variegata* and the parchment-shelled *E. macularius* did not differ. Nonetheless, because *G. variegata* had a significantly higher

Table 2. Effects of embryo stage and species on log 10 coverage (%) of eggs by the YS and CAM. Results of overall ANCOVAs are presented in the upper panel and the lower panel presents results of pair-wise comparisons of CAM development

Coverage	F (df)	P-value	R ²
YS: Overall	28.1 (5, 33)	<0.000	0.811
YS: Stage	96.3 (1, 33)	<0.0001	
YS: Species	35.2 (2, 33)	<0.0001	
YS: Stage*Species	7.6 (2, 33)	0.0019	
CAM: Overall	32.1 (7, 54)	<0.0001	0.806
CAM: Stage	172.2 (1, 54)	<0.0001	
CAM: Species	10.9 (3, 54)	<0.0001	
CAM: Stage*Species	8.9 (3, 54)	<0.0001	
Ct-Gv: Species	0.9 (1, 23)	0.34	
Ct-Gv: Stage*Species	3.4 (1, 23)	0.08	
Ct-Em: Stage*Species	16.4 (1, 29)	0.0003	
Ct-Ol: Stage*Species	23.3 (1, 24)	<0.0001	
Gv-Em: Species	29.7 (1, 30)	<0.0001	
Gv-Em: Stage*Species	1.0 (1, 30)	0.33	
Gv-Ol: Stage*Species	4.3 (1, 25)	<0.05	
Em-Ol: Stage*Species	6.4 (1, 31)	<0.02	

Ct, *Chondrodactylus turneri*; Gv, *Gehyra variegata*; Em, *Eublepharis macularius*; Ol, *Oedura lesueurii*.

Results of pair-wise comparisons of YS development are presented in the text. Species contrasts are not presented when the interaction term was significant.

intercept than *E. macularius*, their CAMs reached 100% coverage at substantially different stages. Similarly, while *E. macularius* had a higher regression slope than *O. lesueurii*, CAM coverage converged as development progressed. Overall, rigid-shelled species completed CAM development at earlier stages (35–36) than parchment-shelled species (stages 39–40).

DISCUSSION

Parchment-shelled eggs are characteristic of most squamates, including about 200 species that comprise the basal clades of gekkotan lizards. Evolution of the rigid-shelled egg is thus a novel innovation (sensu Hallgrímsson et al., 2012) associated with the clade of approximately 1,450 species of gekkotans (Gamble et al., 2012). The numerical success of the derived phyllodactylid, gekkonid, and sphaerodactylid geckos is a conundrum as the rigid-shelled egg comes with a demographic cost (Pike et al., 2012). Gekkotans that lay rigid-shelled eggs produce small eggs relative to female body size. A putative reason is that rigid-shelled eggs are spherical, a shape that minimizes the cost of providing calcium for the shell. Because eggs must pass through the pelvic aperture, however, spherical eggs have smaller volumes than parchment-shelled eggs that have the same diameter but are elongate

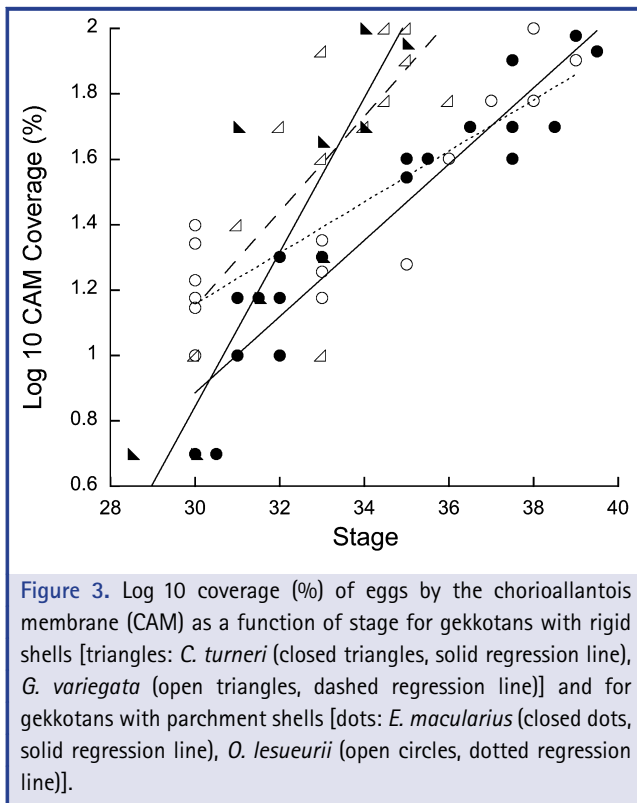


Figure 3. Log 10 coverage (%) of eggs by the chorioallantois membrane (CAM) as a function of stage for gekkotans with rigid shells [triangles: *C. turneri* (closed triangles, solid regression line), *G. variegata* (open triangles, dashed regression line)] and for gekkotans with parchment shells [dots: *E. macularius* (closed dots, solid regression line), *O. lesueurii* (open circles, dotted regression line)].

(Kratovichil and Frynta, 2006). Moreover, gekkotans that lay rigid-shelled eggs produce hatchlings that are small relative to egg size (Pike et al., 2012). Rigid-shelled eggs must contain all the water needed for embryonic development. Thus, for their initial volume, rigid-shelled eggs have relatively lower amounts of lipids and proteins than parchment-shelled eggs that absorb water from the environment after oviposition (Belinsky et al., 2004). In aggregate, these observations suggest that the rigid-shelled egg reduces reproductive output. What are possible benefits of rigid-eggs that could offset this cost? Because rigid-shelled eggs contain all the water they require for development, they can be placed on or in a diversity of dry substrates (Pike et al., 2012). Egg mortality from desiccation should be virtually non-existent and the rigid highly mineralized shell may additionally provide protection from microbial pathogens and from predators such as small invertebrates (Moreira and Barata, 2005). An increase in egg survival may thus offset the reduction in reproductive output associated with the rigid-shelled egg.

In parallel with their demographic cost, the rigid-shelled egg is also associated with a 1,000-fold reduction in gas permeability (Deeming and Thompson, '91; Andrews, 2012). Low permeability of the shell to oxygen may impose a physiological cost on embryonic development per se. To test this idea, we first compared embryonic VO_2 of *G. variegata*, a species that produces rigid-shelled eggs, with that of *O. lesueurii*, a species that produces

parchment-shelled eggs. Metabolic rates differed only at 27.7°C but not at higher experimental temperatures where the oxygen should have been most limiting to embryos in rigid-shelled eggs (Fig. 1). Rather than reflecting oxygen availability, metabolic rates of *G. variegata* and *O. lesueurii* embryos appear to reflect adaptation to their normal incubation environments. *G. variegata* eggs normally experience higher and more variable nest temperatures than *O. lesueurii* eggs. Female *G. variegata* place their eggs on the soil under logs in areas exposed to the sun much of the day. Mean temperatures of two *G. variegata* nests over two days were 24.2 and 25.2°C with a range of 20.0–27.4°C (Bustard, '69). An even wider range (17–38°C) was reported by Henle ('90). In contrast, *O. lesueurii* eggs are laid in deep crevices of large rock outcrops; mean temperature of nests at a site south (i.e., cooler) than the site where *O. lesueurii* were collected for our study was 22.7°C with a range of only 21.0–24.5°C (Pike et al., 2010). We also tested the oxygen limitation hypothesis using a comparative biology approach. Published values of VO_2 max of rigid-shelled gekkotans did not differ from those of parchment-shelled gekkotans plus scincids and VO_2 max of gekkotans did not differ from those of scincids overall. These results do not support the idea that embryonic metabolic rates of gekkotans with rigid shells are limited either proximately or evolutionarily by low oxygen diffusion through the shell. This conclusion does not mean, however, that development is unaffected by increased diffusion resistance to oxygen across the eggshell.

We also tested the idea that embryos would exhibit adaptive changes in development that compensate for low oxygen diffusion through the rigid-shelled egg. To do this, we focused on the YS and the CAM, the extra-embryonic membranes associated with oxygen transport from the environment to the embryo (Lomholt, '84). For typical oviparous squamates, the terminal/definitive YS (Stewart and Florian, 2000) begins to expand prior to oviposition. At this time the oviduct is relatively hypoxic because the embryo does not have a vascular system and the rate of oxygen diffusion through the fluid-filled the spaces in the shell is substantially slower than it would be through air-filled spaces in the shell after oviposition. The CAM is formed about the time of oviposition (Fig. 3) when the allantois contacts the chorion and subsequently expands around the surface of the egg as a vascularized conjoined membrane. At, or shortly after oviposition, oxygen diffusion through the shell increases because spaces in the shell are filled with air. Because the rate of diffusion of oxygen through rigid-shells should be low relative to that of parchment shells both prior to and following oviposition, we predicted shifts in the timing of YS and CAM development would compensate for, at least in part, the relative low diffusion rates of oxygen through rigid-shells.

This prediction was supported. Not only is YS initiated at earlier stages, but the YS is more expansive at the time of oviposition for species with rigid shells (stages 25–26) than those with parchment shells (stage 28; Fig. 2). The YS is the only vascularized extra-

embryonic membrane while eggs are in the oviduct and, therefore, the earlier development of YS would enhance oxygen transport to embryos in rigid-shelled eggs (Lomholt, '84). For both shell types, the YS reached its maximum extent at stages 32–33. Because, therefore, the YS is more advanced at earlier stages for species that lay rigid-shelled compared to species that lay parchment-shelled eggs, the rate of expansion of the YS of the former was slower relative to stage and absolute time (because the incubation period is relatively long for species that lay rigid-shelled eggs). Observations on CAM development also indicated accelerated development in rigid- versus parchment-shelled eggs. Expansion of the CAM is initiated about the same stage regardless of shell type. At stage 30, for example, CAM area was 10–15% of the egg surface area for all four species. Thereafter, the CAM expanded much more rapidly and reached maximum coverage sooner for the species with rigid- (stage 35–36) than parchment-shelled eggs (stage 39–40). In a parallel example of adaptive variation in development, the CAM of *Sceloporus scalaris*, a species that normally retains eggs in the oviduct to advanced embryonic stages, is more expansive at the same embryonic stages than the CAM of *Sceloporus virgatus*, a species that oviposits earlier relative to stage (Andrews, '97).

Our theoretical calculation of the internal PO_2 of a rigid-shelled egg indicates that resistance of the rigid shell to diffusion of oxygen reduces internal PO_2 to 3 kPa below ambient PO_2 . A difference of this magnitude can reduce developmental rates of squamate embryos (Parker et al., 2004; Stahlschmidt and DeNardo, 2008). Nonetheless, embryonic metabolism has been conserved across eggshell types. If this is correct, “normal” embryonic metabolic rates of gekkotan embryos in rigid-shelled eggs are the result of adaptive variation in mechanisms that allow embryos to compensate for relatively low oxygen diffusion per se. For example, we found striking differences between rigid- and parchment-shelled gekkotan eggs in the pattern and rate of development of the YS and the CAM that should provide more oxygen to embryos in rigid-shelled eggs. Because these membranes reach their full extent before the end of development, we suggest that changes in YS and CAM development may be accompanied by other adaptations such as alterations to blood chemistry and vascular density of the YS and CAM.

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