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PATTERNS OF EMBRYONIC NUTRITION AND CHARACTERISTICS OF CHORIOALLONTOIC PLACENTA IN A VIVIPAROUS RACERUNNER LIZARD, *Eremias multiocellata*: A PRELIMINARY STUDY

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In this study we observed the embryonic development processes and compared the weight of eggs with neonates to identify the form of nutritional provisioning in *Eremias multiocellata*. We also used light microscopy to determine the features of chorioallontoic placenta during late stages of gestation. Neonatal dry weight was approximately 10.3% smaller than egg dry weight. Chorioallantoic membrane and uterine epithelium exhibited an indirect apposition without any fusion. No specialized region, placentome, has been found for nutrition transfer between these membranes. The chorioallontoic placenta of *E. multiocellata* is consistent with the simplest and most common type, Weekes type I. We infer that *E. multiocellata* is predominately lecithotrophic, meaning that nourishment for embryonic development is primarily supplied by the yolk. This kind of embryonic nutrition confirms the pattern found in most squamates with a simple placenta. This simple placenta adds anatomical evidence to understand the weak immune interaction between matrix and fetus in *E. multiocellata*. Finally, we discussed the implications for interpreting the evolution of viviparity and placentation.

Keywords: Chorioallontoic placenta; Lecithotrophy; Placentotrophy; Viviparity; *Eremias multiocellata*.

INTRODUCTION

Squamate reptiles are unusual among vertebrate lineages in that viviparity (live-bearing) has evolved independently 115 times within lizards and snakes, out of only ~141 origins in vertebrates (Sites et al., 2011). In general, eggs of oviparous (egg-laying) species are oviposited at an early embryonic stage and complete development outside the female. Most of the nutrients required by the developing embryo in oviparous species are encased within the egg shell (Adam et al., 2007). In contrast to oviparity, embryos of viviparous species complete development within the female reproductive tract and are born as free-living neonates. The viviparous species have various ways to support developing embryos as a result of multiple independent evolution of viviparity (Blackburn, 2000).

Pattern of embryonic nutrition is defined by the source of nutrients (Blackburn et al., 1985) and two sources are distinguished for squamate reptiles, yolk and other non-yolk maternal sources (Blackburn, 1994). Lecithotrophy denotes that yolk provides most of the nutrients for embryonic development, whereas matrotrophy is nutrient provisioning from other maternal sources (Stewart and Blackburn, 1988). The source of matrotrophic nutrients for squamate reptiles is the female reproductive tract, the oviduct. Exchange between the oviduct and the embryo is mediated by extraembryonic membranes, principally the yolk sac and chorioallantoic membrane, tissues shared by all amniotes. This pattern of matrotrophy is termed placentotrophy. In contrast to a simple placenta for lecithotrophy, the transfer of nutrition in placentotrophy requires a more specialized one (Blackburn, 1993).

A simple placenta is defined as a placenta that has a relatively smooth uterine lining and has little intimate contact between the embryonic tissues and maternal tissues (Weekes, 1935). During pregnancy, the placenta undergoes structural and functional changes associated with the changing metabolic demands of the growing embryo (Weekes, 1935). One placental region is the chorioplacenta, which is rare to observe and appears only for a short time, if at all (Mossman, 1937). A second primary placental region is called the omphaloplacenta, which is

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also known as the yolk sac placenta. It is typically formed at the abembryonic pole during the early stages of embryonic development and broadly describes the apposition of the bilaminar yolk sac to the uterine epithelium (Stewart and Blackburn, 1988). Finally, the chorioallantoic placenta (allantoplacenta) is present later in development at the embryonic pole and is formed by apposition of the chorioallantoic membrane to the uterus (Stewart, 1992). This is the organ of respiratory exchange between the mother and embryo (Blackburn, 1993). Both the yolk sac and the chorioallantoic placenta demonstrate a wide range of morphological diversity and exhibit varying degrees of complexity across squamates (reviewed in Stewart and Thompson, 2000).

Racerunner lizards of the genus *Eremias* (family Lacertidae) are the dominant reptiles in the deserts and steppes of Central Asia. The significance of this group is based on the presence of both oviparous and viviparous species. Viviparity in racerunners might have arisen more than once, but one recent study did demonstrate that the viviparous species form a robust clade within the subgenus *Pareremias* (Guo et al., 2011). The Multiocellated Racerunner, *E. multiocellata*, is the species in which viviparity was first discovered in the genus *Eremias* (Shnitnikov, 1928). It occurs in Northwestern China and adjacent areas including Kazakhstan, Kyrgyzstan, Mongolia, and Russia (Zhao et al., 1999; Guo et al., 2011). Szczerbak (1974) speculated that this species was lecithotrophic on the basis of the relatively large size of oviductal eggs. So far no more information about pattern of its embryonic nutrition has been available. Recently, a weak immune interaction between matrix and fetus in *E. multiocellata* was inferred from comparisons of MHC expression between the pregnant and non-pregnant stage through RNA-seq (Yuan, 2014). Judging from this observation, we predict that *E. multiocellata* may have a simple placental structure.

As highlighted by Blackburn (1994), comparisons of the relative size of the ovum with the fetus can reveal little about nutrient sources in relatively lecithotrophic forms. We consider the speculation of Szczerbak (1974) to be very tentative. Thus, the starting point of our work is to test Szczerbak's (1974) hypothesis that *E. multiocellata* is lecithotrophic, for which yolk provides the major source of nourishment for embryonic development. In addition, we will describe the apposition of the fetal and maternal tissues during late gestation, so as to explore the characteristics of its chorioallantoic placenta. This work represents the first characterization of chorioallantoic placenta in any racerunner species of the genus *Eremias*. The results herein will be helpful to enrich our understanding of the evolution of viviparity and placentation.

MATERIAL AND METHODS

Animal collection. Lizards were collected during April of 2014. Viviparous female *Eremias multiocellata* were collected from Jingyuan County, Gansu Province, China (36°32′20.38″ N 104°41′30.73″ E). The lizards were returned to Chengdu Institute of Biology and kept in aquaria containing sand from their site of collection. The room temperature was 21 – 24°C, and heat was provided by a 60-W incandescent light bulb suspended over one end of each aquarium, which created a thermal gradient from 21 – 35°C, allowing lizards to thermoregulate for 10 h a day. The light regime followed that of the local environment. Lizards were fed meal worms (*Tenebrio molitor*) three times per week and provided with water *ad libitum*.

Dissection and conceptus weight measurement. A total of 12 female samples were used to collect data on gestational changes in dry weight and wet weight of the conceptus, respectively (Table 1). The staging scheme was based on Dufaure and Hubert (1961) where zero is fertilization and 40 is birth. For *E. multiocellata*, the embryonic stages were roughly assessed as three periods: early gestation with features corresponding to embryonic stages 30 – 31 of Dufaure and Hubert (1961), mid-gestation with features corresponding to embryonic stages 34 – 35, and late gestation with features corresponding to embryonic stages 38 – 40. One of the females had oviductal eggs with no visible embryonic development. Eggs from the remaining nine females had progressed to early embryogenesis and represented three periods: early, middle to late gestation in three females, respectively (see Table 1). Following parturition, neonates from two females were also weighted.

Females were measured for snout-vent length (SVL) to the nearest 0.1 mm using digital caliper. The SVL from all female specimens were undertaken with the ANOVA tests to find whether there is significant difference between them. Pregnant lizards were euthanized by an intrathoracic injection of 0.2 ml sodium pentobarbital (6 mg/ml) at various times during May – June 2014. The excised eggs were separated from the female coelom, and weighed for wet weight to the nearest 0.1 g on an electronic balance and weighed the dry weight after lyophilized. The zygote was described to document fertilized ovum at stage 0. Pictures were taken to record the primordial state of the eggs with Nikon D3100 camera. Meanwhile, stages of embryonic development were assessed, and voucher specimens were retained for later confirmation.

The wet weight and dry weight data from different females within the same period were undertaken with the ANOVA tests. Given that no significant differences were found among different pregnancy periods, mean value and standard error of the mean (SEM) were calculated for all samples afterwards. Eggs and neonates were compared to assess patterns of nutritional provision to embryos. In general, a substantial decrease in dry weight of the conceptus of viviparous forms is assumed to indicate lecithotrophy (Blackburn, 1994; and references therein). By the same token, placentotrophy has been assumed when dry weight of the neonate greatly exceeds or even is equivalent to that of the egg early in development. As a supplemental strategy, data on gestational changes in wet weight of the conceptus of viviparous forms can be broadly indicative of extremes of lecithotrophy or placentotrophy (Thompson, 1981).

Tissue and embryo harvest. Eggs with intact maternal oviducts wrapped were excised from three females in late gestation. The tissues were stabilized in Bouins' Fluid, dehydrated in increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin (Blackburn and Vitt, 2002). The tissues were sectioned transversely with regard to the long axis of the uterus. Paraffin-embedded uterine tissues with eggs were sectioned at 6 μm thickness using a microtome. The sections were mounted on slides and prepared for staining with hematoxylin and eosin (HE) using standard methods (Humason, 1979). All slides were examined with a Leica-DMRA2 light microscope. Images were captured using a Leica DFC 490 digital camera and LEICA APPLICATION SUITE image software.

RESULTS

General observations. As well with its oviparous relatives, the female's abdomen obviously expanded in size in mating season. The eggs lay in oviducts and shaped as ellipsoid. The mean size of eggs was about 8 – 10 mm in vertical axis, with 4 – 5 mm in horizontal axis. No remnant eggshell has been observed to encase the egg among all the specimens. The eggs were orange in color with homogenous texture before the embryo development (Fig. 1*A*). However, some yellowish deposits existed in the bottom of these eggs which seemed to be the isolated yolk mass. The artery formed by oviduct was adjacent to the mesometrium and ran longitudinally along the dorsum of the egg. Several branches of vein extended from capillaries in the tissue, whereas all these vessels were very thin and hard to discern.

In early gestation, the embryo had begun to develop, and the egg became two parts: yolk mass and embryo. The artery and vein of the maternal oviduct covered a greater surface area of the egg (Fig. 1*B*). The membranes which wrapped the egg were hyaline. The fetus was small and the amnion was filled with fluid. Fetal skin was permeable and pink-and-white; no stripes as adults were shown. Notably, the fetal eyespots formed with a black tube. Fetal blood vascular system had also developed to work. Interestingly, the eggs in the same matrix seemed not to develop synchronously.

In mid-gestation, the fetal body size increased, while yolk mass significantly decreased (Fig. 1*C*). The membranes were hyaline and oviduct's vessels remained to cover the eggs. Fetus was easy to recognize as head, trunk, limbs and tail. In some fetuses, feeble heartbeat could be noticed. In late gestation, the yolk sac was small with little yolk left and occupied at the abembryonic pole.

TABLE 1. The Amount and Weight of Oviductal Eggs and Neonates in Pregnant Specimens

Stage	Voucher number	Weight of females, g	Snout-vent length of females, mm	Litter size	Wet weight of eggs or neonates in each female, g	Mean dry weight in each female, g
Prior	GUO2881	5.1	59.0	4	0.4; 0.4; 0.41; 0.40	0.39; 0.39; 0.38; 0.39
Early (stages 30-31)	GUO2848	4.8	59.7	3	0.41; 0.43; 0.42;	0.38; 0.39; 0.41
	GUO2849	5.3	60.0	4	0.42; 0.43; 0.42; 0.41	0.37; 0.40; 0.38; 0.39
	GUO2856	5.6	58.9	4	0.42; 0.43; 0.41; 0.43	0.39; 0.40; 0.37; 0.40
Middle (stages 34-35)	GUO2858	6.1	60.0	5	0.46; 0.48; 0.48; 0.50; 0.48	0.41; 0.40; 0.42; 0.41; 0.41
	GUO2860	5.5	58.8	4	0.45; 0.47; 0.46; 0.46	0.40; 0.39; 0.41; 0.40
	GUO2861	5.6	59.5	4	0.47; 0.46; 0.47; 0.48	0.40; 0.41; 0.43; 0.42
Late (stages 38-40)	GUO2865	5.4	58.9	3	0.55; 0.58; 0.56	0.42; 0.43; 0.42
	GUO2866	5.3	58.8	3	0.54; 0.55; 0.57	0.4; 0.43; 0.43
	GUO2867	5.5	60.0	3	0.54; 0.59; 0.58	0.44; 0.43; 0.45
Postpartum (neonates)	GUO2873	4.0	60.7	4	0.42; 0.41; 0.43; 0.42	0.33; 0.37; 0.35; 0.34
	GUO2880	3.8	59.5	4	0.43; 0.44; 0.42; 0.42	0.35; 0.36; 0.34; 0.36

Fig. 1. Morphology of developing embryos at different stages: *A*, no remnant eggshell before embryo development, near postzygote stages; *B*, the vessels develop at early pregnancy (stages 30 – 31); *C*, yolk mass significantly decreases at middle pregnancy (stages 34 – 35); *D*, the fetus develops completely at late pregnancy (stages 38 – 40).

Vessels still existed and covered the whole egg. The embryo had developed into a complete fetus with eyes closed (Fig. 1*D*). Fetus was surrounded with amniotic fluid. Fetal heartbeat was much stronger. Stripe turned into grey for the neonates.

Measurement data. Mean snout-vent length for the sampling female *E. multiocellata* was 59.4 ± 0.2 mm ($N = 12$) (Table 1). The ANOVA tests indicated insignificant difference in SVL of all those female samples ($P = 0.58$) at the 5% level of significance for difference. Litter size, based on number of oviductal eggs and number of neonates, ranged from 3 to 5 (Table 1). The analysis undertaken with the application of the ANOVA showed an insignificant variation among eggs from different females within each period (Table 2). As shown in Table 3, mean wet weight of the whole eggs increased significantly along with the developmental stage. Mean dry weight of the whole eggs were as follows: 0.39 ± ± 0.01 g ($N = 4$) near postzygote stage, 0.39 ± 0.01 g ($N = 11$) in early gestation, 0.41 ± 0.01 g ($N = 13$) in mid-gestation, 0.43 ± 0.01 g ($N = 9$) in late gestation. Wet and dry weight of neonates were 0.42 ± 0.01 g and 0.35 ± 0.01 g ($N = 8$), respectively, indicating no increase compared with eggs in the early pregnancy (Table 3). However, the mean dry weight of neonates was approximately 10.3% less than the eggs in early gestation.

Placental membranes. The arrangement of extraembryonic membranes of *E. multiocellata* in late gestation was shown in Fig. 2. The amnion covered the developing embryo to form the amniotic cavity. The amnion contacted the internal surface of the inner allantoic membrane in most regions (Fig. 2, arrowhead). The allantoic cavity was between the chorioallantoic membrane and inner allantoic membrane. The chorioallantoic placenta was formed through apposition of the vascularized chorioallantois to the uterine (Fig. 3). Both the

TABLE 2. ANOVA Measurements of Variables of Female Specimens within ONE STAGE (values are *P*-values).

	Early	Middle	Late	Postpartum
Wet weight	0.911 (NS)	0.062 (NS)	0.607 (NS)	0.278 (NS)
Dry weight	0.737 (NS)	0.123 (NS)	0.173 (NS)	0.628 (NS)

NS, not significant with $P > 0.05$.

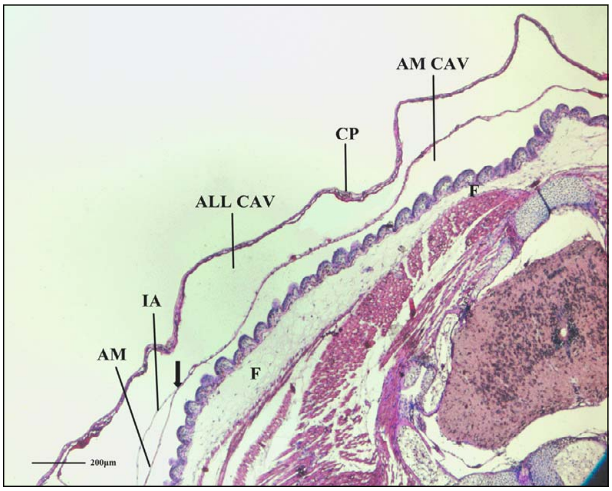

Fig. 2. Arrangement of extra-embryonic membranes in late pregnancy at low magnification in *Eremias multiocellata*; 50×. The amnion contacts the internal surface of the inner allantoic membrane in most regions (arrowhead): AM, amnion; IA, inner allantoic membrane; F, fetus; ALL CAV, allantoic cavity; AM CAV; amnion cavity; CP, chorioallantoic placenta.

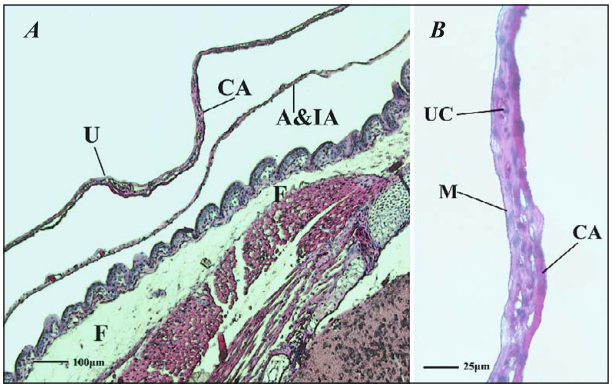

Fig. 3. *A*, Chorioallantoic placenta consists of the chorioallantoic membrane in apposition to the uterine; 100×; *B*, chorioallantoic placenta at higher magnification; 400×; U, uterine; CA, chorioallantoic placenta; A&IA, amnion fused to inner allantoic membrane; UC, Uterine capillaries; M, uterine muscle; F, fetus.

chorioallantois and uterus were well vascularized. Placentome region, which is a specialized region of hyperplasia, hypertrophy and interdigitation, was not observed in chorioallantoic placenta of *E. multiocellata*. Likewise, there were no specialized tissues that could be recognized on uterine epithelium except uterine capillaries (Fig. 3*B*). The outer surface of chorioallantois and the uterine epithelium formed an indirect apposition without any fusion, indicating no intimate contact between them. Most parts of the maternal-fetal interface were smooth, lacking in ridges or folds. Both the two membranes were very thin and hyaline. In *E. multiocellata*, thickness of uterine epithelium was about 15 µm and chorioallantoic membrane was about 10 µm (Fig. 3*B*). This kind of thickness just corresponded to a normal cell in size.

DISCUSSION

Patterns of embryonic nutrition. For *E. multiocellata*, the neonates decreased approximately 10.3% in dry weight when compared with the eggs in early gestation (Table 3). This is in remark contrast to some placentotrophic lizards like *Pseudemoia entrecasteauxii*. As highlighted by Stewart and Thompson (1993), for *P. entrecasteauxii*, neonatal dry weight was 168% greater than egg dry weight and neonatal wet weight was 396% greater than egg wet weight. As proposed by Blackburn (1994), a substantial decrease in dry weight of the conceptus of viviparous forms is assumed to indicate lecithotrophy. Thus, in support of Szczerbak's hypothesis, we infer that *E. multiocellata* is predominantly lecithotrophic, yolk of the ovum providing most nutrients for development.

There appears to be an increase in dry weight through development, until the final stage. This would be unusual for a lectithotrophic lizard. There are two potential explanations. First, one of the issues may ascribe to the small sample size in the present study. If increasing the sample size, a robust statistical analysis may indicate no significant difference among all categories. Second, we can't preclude the possibility of placental transfer contributing to embryonic nutrition in *E. multiocellata*. While the majority of viviparous squamates are lecithotrophic, it is common for these species to exhibit some degree of placental transfer of inorganic and organic nutrients as well (e.g., Swain and Jones, 1997; Thompson et al., 2000; Speake et al., 2004). A more detailed nutrient component analysis will contribute to a more reliable understanding of embryonic nutrition patterns. Specifically, vessels formed by uterine epithelium begin to wrap the egg from the beginning and last through all the gestation periods. With the presence of such a strong vessels network, the

TABLE 3. Statistical Values of Wet and Dry Weight in Different Stages (values are mean ± S.E.M.).

	Prior (*N*)	Early (*N*)	Middle (*N*)	Late (*N*)	Postpartum (*N*)
Wet weight, g	0.41 ± 0.01 (4)	0.42 ± 0.01 (11)	0.47 ± 0.01 (13)	0.56 ± 0.01 (9)	0.43 ± 0.01 (8)
Dry weight, g	0.39 ± 0.01 (4)	0.39 ± 0.01 (11)	0.41 ± 0.01 (13)	0.43 ± 0.01 (9)	0.35 ± 0.01 (8)

wet weight may increase in late pregnancy (Table 3). Sure, this interpretation also needs further corroboration with statistical analyses by increasing the sample size. With the current data, we cannot rule out the latter alternative hypothesis. Thus, we can only infer that the fetal nutrition may follow the lecithotrophic way which means yolk of the ovum provides the primary nourishment of the developing embryo.

Information on embryonic nutrition among viviparous squamates is still scant, particularly in view of the great number of viviparous species. Nonetheless, as reviewed by Stewart (2013), more and more studies support an emerging perspective that placentotrophy may occur in all viviparous Squamata species, regardless of the quantity of yolk present at ovulation. For example, a recent study revealed that viviparous snakes with simple placenta are capable of transporting diet-derived amino acids to their offspring during gestation, possibly via placentation (van Dyke and Beaupre, 2012). Thus, to increase our understanding of the patterns of embryonic nutrition in *E. multiocellata*, it is necessary to perform composition analysis of eggs and neonates (Blackburn, 1994, 2000), and to reveal the capacity for placental transfer by radiotracer studies.

Chorioallontoic placenta type. Over the past century, four major types of chorioallontoic placenta have been defined in squamate reptiles and are differentiated by levels of morphological complexity. Weeks (1935) described the first three types and a fourth has been proposed by Blackburn (1993). The simplest and most common type of chorioallontoic placenta, Weekes type I, is characteristic of indirect apposition of the chorioallantoic membrane to the uterus without any anatomical specializations in either structure. A reduced shell membrane encasing the embryo is usually present and it is this structure that lies in contact with the uterine epithelium. In type II, uterine vessels lie on raised folds apposed to enlarged chorionic epithelial cells. In type III, an elliptical shaped area adjacent to the supporting mesentery of the uterus, the mesometrium, contains vascularized uterine folds and enlarged uterine and chorionic epithelial cells. In other words, in types II and III, there is emergence of the placentome, a specialized region that forms at the embryonic pole and brings the chorionic epithelium and uterine epithelium in direct apposition. The type IV chorioallantoic placenta is the most complex and has been only described in the Neotropical skink genus *Mabuya* (Blackburn et al., 1984; Blackburn, 1993). This type of chorioallantoic placenta undergoes extensive transformation and exhibits invagination of the chorioallantoic membrane by uterine endometrium forming interlocking projections.

In *Eremias multiocellata*, the chorioallantoic placenta has an oval outline and appears as an opaque region at the dorsal pole of the egg. We found that the vascularized uterine epithelium and chorioallantoic membrane formed an indirect apposition without any fusion. On the one hand, we can't see emergence of any structure of placentome. Thus, we infer that the chorioallantoic placenta of *E. multiocellata* is consistent with a Type I placentation as documented by Weekes (1935). On the other hand, we haven't observed any remnant of the shell membrane during the gestation in *E. multiocellata*. The absence of shell membrane is somewhat different from the description of strict Weeks type I. As reviewed by Blackburn (1993), remnants of the shell membrane can be very difficult to see histologically because it is exceedingly thin compared to the oviparous eggshell. The observation that the shell membrane is entirely lacking in the region of the allantoplacenta should be validated with scanning electron microscopy.

Chorioallontoic placentae in viviparous squamates serve several functions including maternal-fetal gas exchange, nutrient transfer, and in at least one skink species, steroidogenesis (Guarino et al., 1998) and immunological protection of the fetus (Paulesu et al., 1995). The major requirement of a placental structure is to aid in gas exchange, which appears to be the primary role of the type I placenta (Blackburn, 1993). In this study, we did not focus on the function of chorioallantoic placenta in *E. multiocellata*. Nonetheless, the simple structure indicates no intimate contact between matrix and fetus, which adds anatomical evidence to explain weak immune interaction between matrix and fetus in *E. multiocellata* (Yuan, 2014). Further studies including structures of yolk sac placenta and developmental changes in the fetal membranes are necessary.

Evolutionary implications. With diverse lines of evidence, a type I placenta has been interpreted as relatively primitive in viviparous Squamata lineages (reviewed in Blackburn, 1993). Some authors speculated that Type I placenta occurs in species that have evolved viviparity relatively recently (Guillette and Jones, 1985). Molecular dating of *Eremias* (Guo et al., 2011) demonstrated that the viviparous group diverged from the oviparous species in subgenus *Pareremias* approximately at 6.3 ± 0.9 million years ago (95% CI, 5.3 – 8.5 Ma). The most recent common ancestor of the viviparous species dated to 5.2 ± 1.1 Ma. Subsequently, rapid speciation events occurred in the viviparous group during the Pleistocene. Thus, our results echo those of Guillette and Jones (1985) in that a simple placenta occurs in species that are thought to have evolved viviparity relatively recently.

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