Reproductive Specializations in a Viviparous African Skink: Implications for Evolution and Biological Conservation

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REPRODUCTIVE SPECIALIZATIONS IN A VIVIPAROUS AFRICAN SKINK AND ITS IMPLICATIONS FOR EVOLUTION AND CONSERVATION

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Abstract.—Recent research on the African scincid lizard, *Trachylepis ivensi*, has significantly expanded the range of known reproductive specializations in reptiles. This species is viviparous and exhibits characteristics previously thought to be confined to therian mammals. In most viviparous squamates, females ovulate large yolk-rich eggs that provide most of the nutrients for development. Typically, their placental components (fetal membranes and uterus) are relatively unspecialized, and similar to their oviparous counterparts. In *T. ivensi*, females ovulate tiny eggs and provide nutrients for embryonic development almost entirely by placental means. Early in gestation, embryonic tissues invade deeply into maternal tissues and establish an intimate "endotheliochorial" relationship with the maternal blood supply by means of a yolk sac placenta. The presence of such an invasive form of implantation in a squamate reptile is unprecedented and has significant functional and evolutionary implications. Discovery of the specializations of *T. ivensi* illustrates why the study of a few convenient "animal models" is no substitute for broad-based studies of biological diversity as directed by phylogenetic considerations. Our study also underscores the value of museum collections to studies of biological diversity.

Key Words.—embryonic development; fetal nutrition; implantation; placenta; placentotrophy; reproductive evolution; reproductive patterns; viviparity

INTRODUCTION

In squamate reptiles, viviparity traditionally has been viewed as a simple pattern that has evolved relatively quickly and through few structural and functional modifications. In viviparous lizards and snakes, embryos are gestated inside the maternal oviduct, but as in oviparous forms, yolk provides most of the nutrients for embryonic development. Simple placentae are formed from apposition of fetal membranes to the oviduct lining, and are mainly responsible for gas exchange. These observations are compatible with the fact that squamate viviparity has evolved frequently (Blackburn 1999a) and, in some cases, at subspecific levels in geologically recent times (Camarillo 1990; Heulin et al. 1993; Qualls et al. 1995; Smith et al. 2001; Surget-Groba et al. 2006). Accordingly, viviparity in squamates is thought to contrast with that of mammals in which nutrients for development are provided by complex placentae with close associations of fetal and maternal tissues.

Since the early 1980s, growing evidence has challenged the view that squamate viviparity is inherently simple and qualitatively different than that of mammals. Notably, placentotrophy has been documented in various scincid lizards (Blackburn and Vitt 1992; Stewart and Thompson 1993; Thompson et al. 1999; Flemming and Branch, 2001; Flemming and Blackburn 2003; Ramírez-Pinilla 2006), where it is accomplished through complex specializations for nutrient transfer (Blackburn 1993a; Flemming and Branch 2001; Jerez and Ramírez-Pinilla 2001; Blackburn and Vitt 2002; Stewart and Thompson 2004, 2009a). Even in these placentotrophic species, however, placentae form through apposition of fetal membranes to the oviduct lining. This situation contrasts markedly with that of many mammals (including humans), in which the conceptus invades deeply into the uterine lining and is bathed directly by maternal blood (Mossman 1987).

In this paper, we shall discuss an extraordinary African species that has converged sharply on reproductive specializations of eutherian mammals. Not only is *Trachylepis ivensi* (Scincidae) highly placentotrophic, but tissues of the developing conceptus penetrate deeply into the maternal tissues through invasive implantation. What results is a degree of physiological intimacy that has never been observed before outside of therian mammals. Existence of these features raises significant functional and evolutionary questions, and has implications for biological conservation and for the use of phylogenetic information to direct studies of biodiversity. Technical details of placental anatomy and development are being presented elsewhere (e.g., Blackburn and Flemming 2009). As our contribution to the symposium volume on reptile

reproduction from the 2008 World Congress of Herpetology, we shall summarize general features of the developmental pattern seen in *T. ivensi* as compared to other squamates and consider their implications for studies on reproductive diversity.

VIVIPARITY AND PLACENTATION
IN GENERALIZED SQUAMATES

Viviparity and fetal nutrition.—Although most squamate reptiles reproduce by laying eggs, viviparity (live-bearing reproduction) is widespread both geographically and phylogenetically. Viviparous squamates are found on every habitable continent and in nearly every conceivable habitat (Blackburn 1982, 1985; Shine 1985). Furthermore, species that exhibit viviparity are scattered among more than 24 families of lizards and snakes and have resulted from over 100 evolutionary origins of this reproductive pattern (Blackburn 1999a, 2000).

Despite the diversity of viviparous squamates, functional similarities are widespread. First, in all viviparous lizards and snakes, females retain developing eggs in their oviducts and give birth to their young. Second, the yolk typically provides most of the nutrients for embryonic development, a pattern retained from oviparous ancestors (Thompson and Speake 2003, 2006). Accordingly, the ovulated yolk typically is large and equivalent or greater in mass than the offspring at birth (Blackburn 1994; Stewart and Thompson 2000). Third, the developing embryos are sustained by placentae that form through apposition of fetal membranes to the oviduct lining. These placentae not only accomplish gas exchange, but also provide at least small quantities of nutrients such as calcium, sodium, and organic molecules (Stewart and Thompson 2000; Thompson et al. 2000; Thompson and Speake 2003). Thus, viviparous squamates are not strictly lecithotrophic, but exhibit some capacity for placentotrophy. The functional similarities are consistent with a scenario in which placentation and incipient placentotrophy evolve simultaneously with viviparity, perhaps through a punctuated equilibrium pattern of change (Blackburn 1992, 1995, 1998a).

Placentation.—An understanding of squamate viviparity requires consideration of the placental membranes through which embryos are sustained during gestation. Placentae are complex organs with multiple components. The literature on squamate placentae is sizeable; however, a number of reviews are available, ranging from summaries written for general audiences (e.g., Blackburn 1999b; Thompson et al. 2004; Thompson and Speake 2006) to reviews that provide details of structure, development, and function (Blackburn 1993b; Stewart and Blackburn 1988; Stewart

1993, 1997; Stewart and Thompson 2000; Thompson et al. 2000, 2006). What follows is a brief summary that outlines some of the most salient placental features in generalized squamates.

Placentae of viviparous vertebrates always have both maternal and fetal components (Mossman 1987). The maternal component in viviparous squamates is formed by the uterine oviduct, which houses the developing eggs. The squamate oviduct is lined by a single layer of epithelial cells, under which lies a thin layer of vascularized connective tissue (Blackburn 1998b; Girling 2002). Glands and other cellular specializations typically are absent during pregnancy; however, uterine vascularity may increase during gestation. The fetal contribution to the placentae is formed by the chorioallantois and yolk sac (Yaron 1985; Blackburn 1993b; Stewart 1993; Thompson and Speake 2006); these give rise to the chorioallantoic placenta and yolk sac placenta (omphaloplacenta) respectively.

The chorioallantoic placenta is the main site of respiratory gas exchange between fetal and maternal blood streams. The chorioallantois is lined by a very thin (squamous) epithelium over the allantoic blood vessels. The apposing uterine epithelium is similarly attenuated over the uterine blood vessels. Both membranes are well vascularized. A remnant of the eggshell (the "shell membrane") commonly persists at the placental interface, although it tends to be so thin by late gestation as to be hard to visualize with light microscopy. Due to attenuation of the uterine and chorionic epithelia, and reduction of the shell membrane, the distance between fetal and maternal blood streams is very small, on the order of a few micrometers. Thus, the chorioallantoic placenta is well suited to its respiratory functions (Blackburn 1993b).

Yolk sac placentae vary in composition and source of fetal vascularization, both during the course of gestation and between species (Stewart and Blackburn 1988; Stewart 1993, 1997). Consequently, generalizations are difficult. However, one notable feature is that tissue lining the external surface of the yolk sac is inherently non-vascular. Although a vascularized choriovitelline placenta forms early in development, it is very limited in extent and disappears as the exocoelom expands (Stewart 1993, 1997; Blackburn and Callard 1997). The omphalopleure can become vascularized internally by the allantois, but this pattern is found only in some viviparous species (Stewart and Thompson, 2000). A second useful generalization is that epithelium lining the yolk sac, as well as that of the oviduct in this region, commonly consists of relatively enlarged (i.e., cuboidal to columnar) cells (Stewart 1993). Moreover, a shell membrane persists at the placental interface, and in some species, can form a thick barrier between fetal and maternal tissues (Weekes 1935; Blackburn 1993a; Stewart 1993). As a result of these features, the
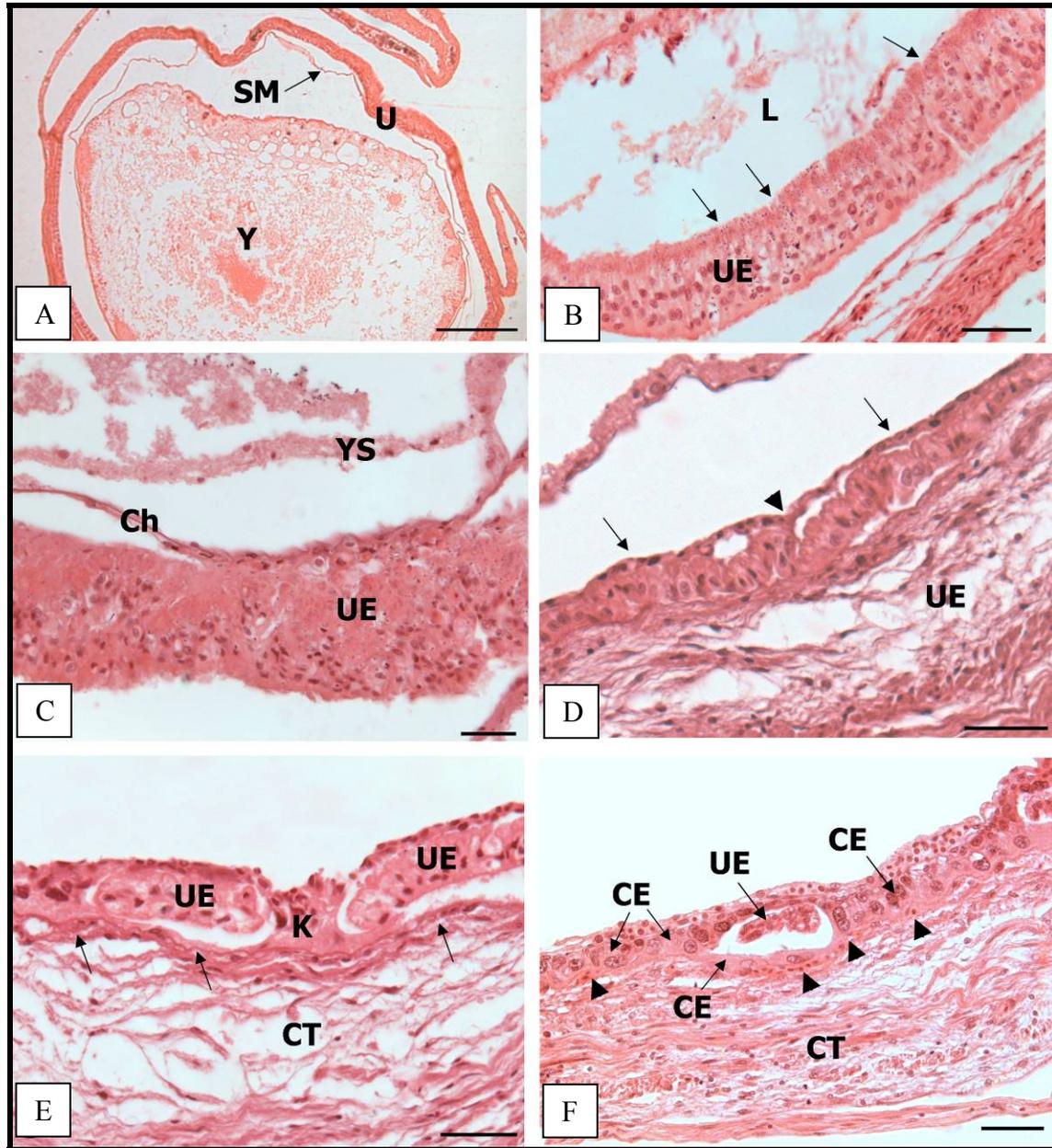
FIGURE 1. Development, placentation, and implantation in the scincid lizard *Trachylepis ivensi*. A) Cleavage-stage egg in the uterus (U). A thin shell membrane (SM) lies between the yolk (Y) and the uterus. B) Uterine epithelium (UE) in the neurula stage, showing basophilic secretory granules (arrows). L = uterine lumen. C) Chorionic placenta in the neurula stage, formed by apposition of the chorion (Ch) to the hypertrophied uterine epithelium (UE). YS = yolk sac splanchnopleure. D) Chorionic placenta, showing sites of attachment (arrows) and penetration (arrowhead) of the uterine epithelium (UE). E) The chorionic epithelium forms knobs (K) that penetrate the uterine epithelium (UE) and invade beneath it, stripping it from the uterine connective tissue (CT). Arrows show bands of deeply invasive chorionic tissue lying beneath the uterine epithelium. F) Endotheliochorial placentation, formed by apposition of chorionic epithelium (CE) to endothelium of uterine capillaries (arrowheads). A piece of shed uterine epithelium (UE) occupies the space between inner and outer layers of the chorionic epithelium. CT = uterine connective tissue. *Scale bars:* A = 250 μm; Figs. B - F = 50 μm.

diffusion distance between fetal and maternal blood systems across the yolk sac placenta tends to be very large, precluding efficient interhemal exchange (Stewart 1993; Blackburn and Lorenz 2003).

Overview.—Three generalizations that apply to both placental types are especially worth noting in the present context. One is that a thin shell membrane persists at the placental interface, preventing direct contact of fetal and

maternal tissues. Second, the uterine epithelium forms an unbroken barrier over the maternal blood vessels, potentially limiting exchange between fetal and maternal blood systems. Third, the fetal tissues never erode the maternal tissues; instead, the uterine lining remains intact.

REPRODUCTION IN *TRACHYLEPIS IVENSI*

Trachylepis ivensi is an elongate lygosomine skink from Central Africa; it has been reported from Zambia, Angola, and the Democratic Republic of the Congo. The genus *Trachylepis* has been established for African lizards that traditionally were referred to the genus *Mabuya* (Bauer 2003). *Trachylepis ivensi* is poorly known and has rarely been collected; thus, our studies are based on histological examination of tissues from museum specimens (Blackburn and Flemming 2009).

In this species, females ovulate minuscule eggs that are only 1 mm in diameter (Fig. 1a). The vitellus appears to be largely depleted of yolk material by the neurula stage. Given the tiny size of the egg and the scarcity of yolk droplets, most nutrients for development must be supplied by means of the placental membranes.

Placental formation.—Soon after ovulation, the uterine epithelial cells begin secreting material into the uterine lumen, where it becomes absorbed by the developing egg. Histological samples show that this secretory material consists of basophilic granules released through merocrine secretion (Fig. 1b) as well as a carbohydrate-rich substance resulting from apocrine secretion. Although a vestigial shell membrane is deposited (Fig. 1a), it disappears by the neurula stage, allowing direct contact between fetal and maternal epithelia.

By the early to mid-neurula stage, the egg is surrounded by two membranes: an avascular chorion and a vascularized choriovitelline membrane. These contribute respectively to chorionic and a choriovitelline placentae. Through the late-neurula stage, continued expansion of the exocoelom converts choriovitelline membrane to chorion (Fig. 1c). However, during the pharyngula stage, swelling of the vascularized yolk sac brings it back in contact with the chorion, expanding the extent of the choriovitelline membrane. As a result, by the late pharyngula stage, the egg is nearly surrounded by a vascularized choriovitelline placenta.

From the neurula stage onward, chorionic epithelial cells begin to invade the uterine tissue (Blackburn and Flemming 2009). They attach to the uterine cells and penetrate between them down to the level of the basement membrane (Fig. 1d). The invading tissue then penetrates beneath the uterine cells, cutting them off from the underlying connective tissue (Fig. 1e). As it invades, the chorionic tissue proliferates, effectively removing and replacing the uterine cells. Consequently, the uterine chamber surrounding the developing embryo becomes lined entirely by fetal epithelium. The fetal epithelial cells lie in direct contact with the uterine blood vessels (Fig. 1f). The fetal epithelial cells are invaginated basally by vitelline blood vessels. As a result, the distance between fetal and maternal blood streams is reduced.

The definitive placenta by the late-pharyngula to early limb-bud stage is therefore a choriovitelline placenta formed through close contact between vascularized fetal and maternal tissues (Fig. 1f). We have not as yet studied tissue samples from later in development, i.e., beyond the limb bud stage. Thus, we do not know whether the choriovitelline placenta persists later in development, or becomes replaced by a chorioallantoic one.

Functional implications.—The reproductive specializations of *Trachylepis ivensi* have several functional implications. First, fetal nutrition in this species is highly placentotrophic. Nutritive material in the tiny yolk (Fig. 1a) may be sufficient to fuel cleavage, but by the early neurula stage, nutrients are being secreted by the uterus and absorbed by fetal tissue (Fig. 1b). Second, the close physiological contact between maternal and fetal tissues established by the limb-bud stage offers a means for direct transfer of nutrients to the developing conceptus (Fig. 1c). The only barrier between maternal blood and chorionic cells is the very thin (endothelial) lining of the uterine blood vessels. Nutrients taken up by the chorionic cells therefore can be passed directly to the fetal blood system. Third, due to the tissue arrangement as well as attenuation of the chorionic cells, the diffusion distance between maternal and fetal blood streams is reduced, allowing efficient gas exchange (Figs. 1e and 1f). Thus, a single tissue arrangement functions in both nutrient transfer and gas exchange. In other placentotrophic lizards, separate regions are specialized for these two functions.

The close arrangement of fetal and maternal tissues in *Trachylepis ivensi* may also have negative consequences. The close contact of fetal and maternal tissues potentially might expose fetal tissues to immunological attack, as well as to the feminizing effects of maternal hormones. How the embryos avoid these effects is unknown.

Comparisons to other squamate.—Extreme placentotrophy is rare among squamates, having been found in only four to six unrelated lineages of scincid lizards on five continents. Placentation has been studied in detail in these lizards, including species from Europe (Blackburn 1993a), South America (Jerez and Ramírez-Pinilla 2001, 2003; Blackburn and Vitt 2002; Ramirez-Pinilla et al. 2006; Vieira et al. 2007), Africa (Flemming

and Branch 2001) and Australia (Stewart and Thompson 2003, 2004, 2009a; Adams et al. 2005). Their placentae show impressive specializations for nutrient transfer, including diverse adaptations for uterine secretion and fetal absorption.

Four significant features distinguish *T. ivensi* from other placentotrophic squamates that have been studied. First is the degree of placentotrophy. At only 1 mm in diameter, the eggs of *T. ivensi* are among the smallest eggs known (see Blackburn et al. 1984; Flemming and Blackburn 2003; Ramirez-Pinilla 2006), and their small size reflects reliance of the embryos on placentotrophic nutrition. The second feature is its pattern of invasive implantation. In the other placentotrophic forms, the uterine tissues lay opposed to the chorionic cells, but are not invaded and entirely replaced by the latter. Third, in *T. ivensi,* the known specializations are features of the choriovitelline placenta. In other viviparous squamates, this type of placenta is transitory and of uncertain significance (Stewart and Blackburn 1988; Blackburn and Callard 1997; Stewart and Thompson 2004; Villagrán Santa Cruz et al. 2005).

The fourth feature has to do with the nature of placental contact. Placentae traditionally are classified by the number of tissue layers lying between maternal and fetal blood systems. Other viviparous squamates have an "epitheliochorial" placenta, so-named because the placental interface is formed by contact between the uterine epithelium and the chorion (Blackburn 1993b). In this type of placenta, up to six layers of cells and tissues lie between fetal and maternal blood systems, in addition to the vestigial shell membrane. In contrast, *T. ivensi* exhibits "endotheliochorial" contact, in which the endothelial lining of blood vessels contacts the chorionic epithelium directly (Figure 1f). This arrangement occurs through loss of the uterine epithelium and underlying connective tissue, and attenuation of other layers. Accordingly, tissues lying between fetal and maternal blood streams consist only of the endothelial lining to the blood vessels and the attenuated chorionic epithelium. The closest parallel to the present situation has been reported in South American *Mabuya*, where individual chorionic cells invade the maternal tissues to contact the uterine blood vessels (Vieira et al. 2007). However, that arrangement is a feature of the chorioallantoic placenta rather than the yolk sac placenta; it also involves invasive cells rather than tissue replacement. Further study is needed to compare the placental arrangement seen in African *T. ivensi* with that of the South American *Mabuya*.

Evolutionary convergence with mammals.— *Trachylepis ivensi* shows some extraordinary reproductive similarities to therian mammals, with regard to its reliance on placentotrophy and the relationship of fetal and maternal placental tissues.

Invasive implantation is a feature previously thought to be confined to eutherian mammals, where it is found in rodents, bats, carnivorans, insectivorans, and primates (including humans; Wimsatt 1975; Enders 1976; Carter and Enders 2004). An "endotheliochorial" arrangement also is characteristic of species of the above mammalian groups, and is a consequence of the invasive implantation (Mossman 1987; Carter and Enders 2004; Wooding and Flint 1994; Carter and Mess 2007). Thus, discovery of these reproductive specializations in *T. ivensi* significantly expands the range of variation known in reptiles to encompass that of eutherian mammals. The situation offers a striking case of evolutionary convergence between amniote lineages whose last shared common ancestor dates back well into the Paleozoic.

IMPLICATIONS FOR STUDIES OF DIVERSITY

Innumerable conceptual and empirical advances in biology have occurred through the study of "model species" that are taken to be representative of larger groups and widespread phenomena. Just as studies on *Drosophila* have contributed to genetics and those on *Caenorhabditis elegans* to developmental biology, squamate reptiles arguably offer a valuable model for understanding viviparity. After all, this reproductive pattern has evolved more frequently in squamates than in all other vertebrates combined (Blackburn 1999a, c), and through recruitment of structures that all amniotes have in common.

However, despite its potential and utility, the model species approach has limitations imposed by diversity, limits that constrain attempts at over-arching generalization (Blackburn 2000, 2006). For example, viviparous matrotrophy in eutherians and placentotrophic skinks shows striking similarities, but the conclusion does not follow that their reproductive patterns evolved in similar ways and under similar selective pressures. On the contrary, phylogenetic analyses suggest that viviparity and matrotrophy evolved by entirely different temporal sequences in mammals and reptiles (Blackburn 2005, 2006). Studies of squamates have potentially can give deep insight into protoadaptations, constraints, and selective pressures, but these factors are likely to vary between clades.

Misapplication of the "animal model" approach to squamates would be risky if it led researchers to focus on a few species assumed (without evidence) to be representative of the entire group of ~7500 species. The known placentotrophic skinks represent about 5 or 6% of the viviparous squamate clades (Blackburn 1999a), scattered on five continents. Two of these clades (the African *Trachylepis ivensi* and *Eumecia anchietae*) occur in geographical regions that are fairly inaccessible to most herpetologists. All of the placentotrophic

species could easily have been overlooked had research focused heavily on other squamate species, such as those that are most accessible to laboratory based investigators. It is worth noting that placentotrophy was discovered among the scincid clades by fortuitous accident, through random samples of diversity (e.g., Giacomini 1891; Weekes 1935; Vitt and Blackburn 1983; Flemming and Branch 2001; Flemming and Blackburn 2003).

The model species approach certainly has its attractions, in view of widespread concerns over species conservation and loss of herpetological diversity. Ironically, however, to focus on a limited number of species models will lead us to overlook the biological diversity we seek to explore and understand. In fact, this very diversity offers one of the many arguments for maintenance of species in the face of human encroachment and ecological devastation.

For explorations of biological diversity, therefore, the most effective approach is to continue sampling biological diversity judiciously, while using phylogenetic information to direct detailed study. Phylogenetic analysis has proven a powerful tool in studies on viviparous lizards. For example, in Australian skinks, it has permitted detailed reconstruction of how viviparity and placentation have evolved in parallel in two distinct but related clades (Stewart and Thompson 2003, 2004, 2009a, b). Similarly, phylogenetic considerations actually contributed to the discovery of placentotrophy in *T. ivensi*. We began investigating this species due to evidence that it may be related to the placentotrophic South American forms (Flemming and Blackburn 2003), an inference that now seems improbable based on its placental anatomy. The likelihood that *T. ivensi* represents an independent origin of placentotrophy and has therefore converged on the South American *Mabuya* makes investigation of its reproductive evolution all the more significant.

This study underscores the value of museum collections in explorations of diversity and reproductive evolution. Our investigations on *T. ivensi* are based entirely on field-fixed museum specimens, yet they have proven quite suitable for histological analysis. Museum specimens also provided the basis for study of placental development in the placentotrophic African skink *Eumecia anchietae* (Flemming and Branch 2001); specimens of this species are exceedingly rare in collections, but vital to the reconstruction of reproductive evolution in placentotrophic skinks. Museum specimens traditionally have been maintained for taxonomic purposes, and now are very useful in DNA-based phylogenetics. While sampling of reproductive tracts from museum specimens is invasive, it does not damage specimens for other sorts of studies. Careful use of museum specimens therefore can contribute immeasurably to our understanding of reproductive diversity and evolution.

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