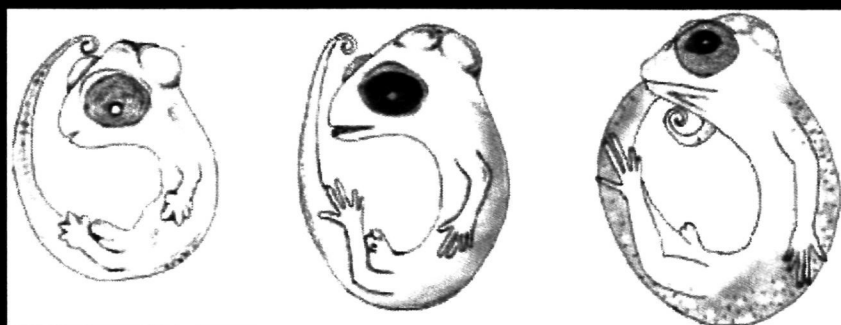


Appendix 3

VOLUME 305A NUMBER 1

JOURNAL OF EXPERIMENTAL ZOOLOGY

PART A
COMPARATIVE EXPERIMENTAL BIOLOGY



WILEY-LISS
ISSN 1548-8969

JANUARY 1, 2006

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Embryonic Gonadal and Sexual Organ Development in a Small Viviparous Skink, *Niveoscincus ocellatus*

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ABSTRACT The majority of research into the timing of gonad differentiation (and sex determination) in reptiles has focused on oviparous species. This is largely because: (1) most reptiles are oviparous; (2) it is easier to manipulate embryonic developmental conditions (e.g., temperature) of eggs than oviductal embryos and (3) modes of sex determination in oviparous taxa were thought to be more diverse since viviparity and environmental sex determination (ESD)/temperature-dependent sex determination (TSD) were considered incompatible. However, recent evidence suggests the two may well be compatible biological attributes, opening potential new lines of enquiry into the evolution and maintenance of sex determination. Unfortunately, the baseline information on embryonic development in viviparous species is lacking and information on gonad differentiation and sexual organ development is almost non-existent. Here we present an embryonic morphological development table (10 stages), the sequence of gonad differentiation and sexual organ development for the viviparous spotted snow skink (*Niveoscincus ocellatus*). Gonad differentiation in this species is similar to other reptilian species. Initially, the gonads are indifferent and both male and female accessory ducts are present. During stage 2, in the middle third of development, differentiation begins as the inner medulla regresses and the cortex thickens signaling ovary development, while the opposite occurs in testis formation. At this point, the Müllerian (female reproductive) duct regresses in males until it is lost (stage 6), while females retain both ducts until after birth. In the later stages of testis development, interstitial tissue forms in the medulla corresponding to maximum development of the hemipenes in males and the corresponding regression in the females. *J. Exp. Zool.* 305A:74–82, 2006. © 2005 Wiley-Liss, Inc.

Sex determination in vertebrates occurs via one of two principal mechanisms/pathways, environmental sex determination (ESD), of which temperature-dependent sex determination (TSD) is the most common, or genetic sex determination. Reptiles have proved to be a very important taxon in understanding sex determination in vertebrates (Pieau et al., '99) because the mode of sex determination shows multiple independent origins—more than any other vertebrate group—making it an ideal group to investigate processes at work during such transitions (for reviews on sex determination, see Bull, '80; Janzen and Paukstis, '91; Pieau et al., '99; Shine, '99).

Vertebrates follow a generalized pattern of gonad differentiation and development (Fox, '77; Morrish and Sinclair, 2002). The gonad initially develops as the genital ridge, a thickening on the

dorsal wall of the coelom. At this stage the gonad is indifferent showing none of the characteristics unique to either male or female, and it contains both the inner medulla and outer cortex regions. This period of bipotentiality occurs in all vertebrates regardless of the sex determining mechanism. However, in species with ESD the gonads are capable of differentiating into either sex depending on the conditions encountered during embryonic development—in oviparous reptiles this

Grant sponsor: Australian Research Council Grant; Grant number: DP0211364; Grant sponsor: Macquarie University New Staff Grants Scheme; Grant sponsor: Macquarie University Grants Scheme.

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Received 2 March 2005; Accepted 26 September 2005

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.a.249.

typically occurs in the middle third of development (see Raynaud and Pieau, '85; Pieau et al., '99; and references therein). Differentiation begins as the germ cells within the gonad congregate in either the inner medulla region in embryonic testis or in the cortex zone of the developing ovary. As development continues, the outer cortex layer in the developing testis regresses while the seminiferous tubules begin to form in the medulla. In the developing ovary, the opposite occurs; the medulla begins to breakdown and the cortex thickens. Later in ovary development, follicles surround the germ cells in the cortex layer, eventually forming oocytes (Raynaud and Pieau, '85; Austin, '88; Wibbels et al., '91; Smith and Joss, '93; Merchant-Larios et al., '97; Greenbaum and Carr, 2001).

The reproductive ducts develop concomitantly with the gonads connecting them to the genitalia. Both male and female reproductive ducts form during embryonic development in both sexes (Pieau et al., '99; Scherer, '99). The male (Wolffian) reproductive duct is initially formed as the collecting duct for the pronephric kidney. This association of the Wolffian duct with the kidneys means it forms early in embryonic development, while the female (Müllerian) reproductive duct does not form until later in embryonic development. The Müllerian duct arises from a region of the coelomic epithelium which covers the cranial part of the embryonic kidney. While the Müllerian duct forms in males, it begins to regress once the testis has differentiated, due to the secretion of an anti-Müllerian hormone. The Müllerian duct degenerates until it is lost in males while it differentiates into the oviducts in females (Raynaud and Pieau, '85; Austin, '88, '89; Wibbels et al., '99; Greenbaum and Carr, 2001).

Most work on embryonic development in reptiles, including gonadal differentiation, has involved oviparous species largely because (1) most reptiles are oviparous, (2) it is easier to assess the timing of embryonic development in eggs than oviductal embryos, (3) it is easier to manipulate embryonic developmental conditions (e.g., temperature) of eggs than oviductal eggs and (4) it was widely believed that modes of sex determination were more diverse (and were consequently explored more vigorously) in oviparous taxa than viviparous taxa because viviparity and ESD/TSD were thought to be incompatible (Bull, '80; Uller, 2003). However, recent evidence suggests that viviparity and ESD/TSD may well be compatible biological attributes (Olsson and Shine, 2001; Robert and Thompson, 2001; Wapstra et al.,

2003, 2004). For example, in both spotted snow skinks (*Niveoscincus ocellatus*) and alpine water skinks (*Eulamprus tympanum*), female basking behavior, i.e., the amount of time female body temperatures are elevated and consequently the temperatures experienced by the developing embryos, affects the sex of her offspring (Robert and Thompson, 2001; Wapstra et al., 2004). Furthermore, facultative sex allocation occurs in female alpine snow skinks (*Niveoscincus microlepidotus*) (Olsson and Shine, 2001) although the mechanism of control remains unclear. Since the thermosensitive period in oviparous species corresponds to the first stages of gonadal differentiation (Pieau, '96), further investigation of these mechanisms requires data on the timing of gonadal differentiation in these viviparous species.

Recent research has opened potential new lines of enquiry into the evolution and maintenance of sex determination mechanisms in reptiles, and in vertebrates in general. However, the baseline information on embryonic development in viviparous reptile species is lacking, and information on gonad differentiation and sexual organ development is almost non-existent. Sexual differentiation and gonad development has not previously been examined in any species of skink (to the best of our knowledge); furthermore, the few embryonic development tables that exist for squamate reptiles (snakes and lizards) are restricted to external development characteristics (Dufaure and Hubert, '61). Here, we provide an embryonic development table based on external characteristics (10 stages) for the spotted snow skink (*N. ocellatus*) and describe the timing of gonad differentiation, the accompanying formation/regression of reproductive ducts and the development of sexual organs (hemipenes).

MATERIALS AND METHODS

Model organism, study site and animal collection

Snow skinks (*Niveoscincus*) are a group of closely related, small ground dwelling skinks largely endemic to the island state of Tasmania, Australia (Melville and Swain, 2000a,b). Spotted snow skinks (*N. ocellatus*) are small (3–10 g adult mass) viviparous skinks common across a wide geographic and climatic range in Tasmania, Australia. Extensive baseline information has been accumulated on this species including on the timing and costs of reproductive events (Jones et al., '97; Wapstra et al., '99; Wapstra and

O'Reilly, 2001; Wapstra and Swain, 2001a,b), geographic and sexual differences in size and age at maturity (Wapstra et al., 2001), annual and geographic variation in life history traits (Wapstra and O'Reilly, 2001; Wapstra and Swain, 2001a) and the effect of maternal basking on offspring phenotype (Wapstra, 2000). Most importantly, as discussed above, this species and others in the genus, have been shown to have biased sex ratios in field and laboratory studies with embryonic sex appearing to be influenced by both maternal temperature and hormone environment during embryonic development (Olsson and Shine, 2001; Wapstra et al., 2003, 2004). In warm lowland sites, reproduction is annual; females ovulate in spring (late September–early October), birth occurs in late-December–mid-January following a relatively long (~3.5 months), weather-dependent gestation (Wapstra et al., '99). In this study, gravid females were collected from the “warm” lowland study site (Orford, Tasmania: previously described in Wapstra et al., '99, 2004; Wapstra and Swain, 2001a) throughout the reproductive cycle. Where possible, embryos were collected from females from which other data were being collected (e.g., Jones et al., '97; Wapstra et al., '99; Wapstra and Swain, 2001b). Where additional embryos from specific developmental times were required, females were sacrificed with an overdose of isoflurane. (This work was carried out with approval of animal ethics from MU (2001/011, 2003/009) and the University of Tasmania (No. 94070).) The embryos were immediately dissected from the females, killed by rapid decapitation and preserved in 10% formalin plus glycerol. Additional data on embryo development were taken from preserved museum specimens from known study sites.

Establishment of embryo development table and histological examination of gonads

Initially, embryos were staged according to a devised table (10 stages) based on obvious external characteristics of the embryos; obvious features identified by Dufaure and Hubert ('61) for *Lacerta vivipara* were used as a guide. Only the development of features which could be observed with the naked eye or by a stereomicroscope were used to design a table suited to *N. ocellatus* (in order that the table could be used subsequently on other lizard species with ease). Detailed illustrations of each of the identified stages were then made, along

with important descriptions of stage-specific features. Measurements of snout–vent length, limb and hemipenis lengths were made on a subsample of the specimens available using an eyepiece graticule.

Following staging, the lower part of the body was removed for histological examination of the gonads and reproductive ducts. The samples underwent standard dehydration through a graded series of ethanols, followed by clearing in a clearing agent (Histochoice®, Ohio, USA). Samples were infiltrated with paraffin wax (Paraplast, Oxford®) overnight in a vacuum oven at 55 °C and 80 Pa before being embedded in fresh paraffin. Transverse sections of 10 µm were cut and routinely stained in Gill's hematoxylin and eosin (HD Scientific®, Sydney, Australia). Sections were examined under a light microscope (Olympus, BX50). Images were captured using a Sony digital camera (DFW-X700) and associated imaging software (BTVpro, version 5.4.1).

RESULTS

Reproductive cycle and timing of embryonic development

The reproductive cycle of *N. ocellatus* has been described previously (Jones et al., '97) including annual and geographic variation in timing of major events such as ovulation and birth (Wapstra et al., '99). Our results here concentrate on the timing of embryonic development. Ten stages of development were identified for *N. ocellatus* (nine during development, with a final stage at, or soon after, birth). Illustrative diagrams and diagnostic features of each of the stages are provided in Fig. 1 and Table 1, respectively, while Fig. 2 provides a generalized timescale of development (taking into account the variation between females within populations, between populations and between years). The development and formation of the limbs, digits and nails were important diagnostic features for the developmental stages as were the pigmentation of the eyes and development of the eyelids (Table 1). Dufaure and Hubert ('61) provide the only other available development table for viviparous lizards, specifically designed for the common European lizard, *L. vivipara*. Through intensive and regular sacrificing of females during gestation under controlled conditions, they were able to identify 40 stages—we have chosen to divide development into 10 generalized stages, which was more appropriate given the samples were sourced from field samples where limited

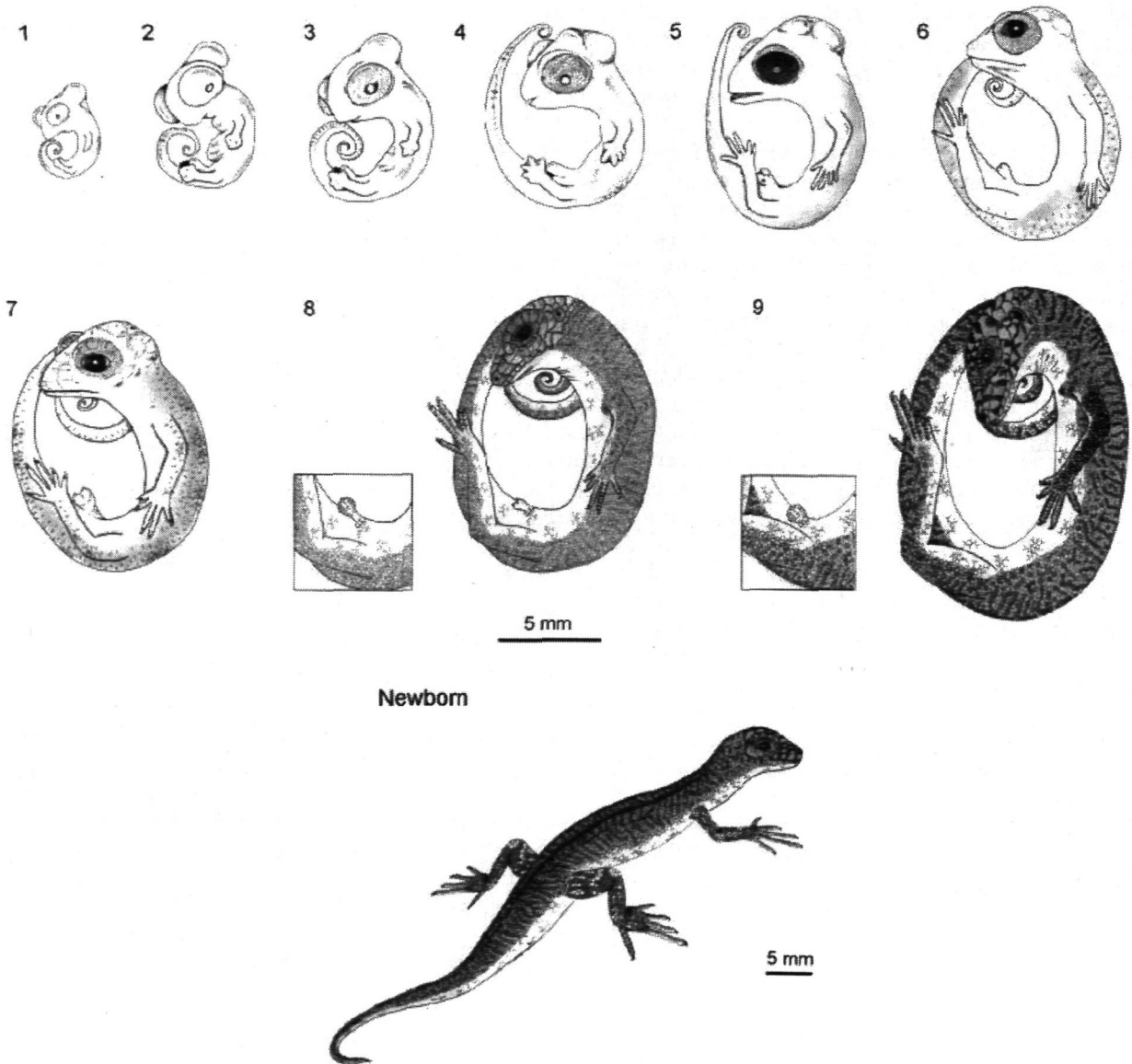


Fig. 1. Illustrative drawings of the 10 stages (nine during development and one at birth) of embryonic development of *Niveoscincus ocellatus* (refer Table 1 for detailed description of external characters).

numbers of females could be sacrificed solely for embryo staging.

Gonad, reproductive duct and hemipenis development

Hemipenis length (total length from base to tip) during embryonic development are displayed in Fig. 3. Hemipenes develop early during development (prior to stage 1) in both sexes; and by stage 5 they start to develop characteristic structure and

shape. Sexual dimorphism in the hemipenes, both in size and shape is first apparent at stage 7 and size continues to diverge in stages 8 and 9. Gonad histology confirmed the sex of each of the embryos for which hemipenis size was measured.

Gonad differentiation proceeds rapidly compared with the concurrent changes in morphology. Accordingly, there is a rapid change from the indifferent gonad of stage 1 (Fig. 4a) to the early stages of differentiation in the stage 2 gonads (Figs. 5a and b). In females, this is characterized

TABLE 1. Stage descriptions of embryonic development in *Niveoscincus ocellatus* including external diagnostic features and measurements

Stage	Distinguishing features	Dufaure and Hubert ('61)	SVL (mm \pm SE)	Anterior limb length (mm \pm SE)	Posterior limb length (mm \pm SE)
1	Limbs differentiated—hind limbs show some signs of comb formation Eyes are large and bulbous and not pigmented except around edge Brain is bulbous with no obvious regions Hemipenes are visible ¹	29	7 \pm 0.05	1 \pm 0.03	1.5 \pm 0.05
2	Comb or paddle formed on both anterior and posterior limbs Slight pigmentation in eyes	30–32	8.5 \pm 0.18	1.5 \pm 0.11	1.8 \pm 0.12
3	Lower jaw begins to form Paddle on limbs becomes wavy, 3 crests Eyes pigmented (brown) ¹	33	11.0 \pm 0.13	2.5 \pm 0.08	3.0 \pm 0.08
4	Circular eyelid visible around edge of eyes, which are less bulbous ² 5 undifferentiated digits visible in wavy paddle 4 regions of the brain visible Lower jaw formed ²	34	12.8 \pm 0.10	3.0 \pm 0.30	3.5 \pm 0.25
5	5 differentiated digits (palms formed) Hemipenes develop in shape Pineal eye presents visible circle	35	18.5 \pm 0.31	4.9 \pm 0.12	6.1 \pm 0.10
6	Pigmentation (tightly packed brown spots) on dorsal surface of body and fainter on limbs ² Ear obvious ² Nails become pigmented Oval eyelids present ⁴ Faint outline of scales Digits annulated	36	19.2 \pm 0.46	5.7 \pm 0.15	7.2 \pm 0.08
7	Strong pigmentation on the dorsal surface of body and limbs Pigmentation (tightly packed brown spots) on head Body scales fully differentiate, large cephalic scales and snout begin to form Nails formed Eyes become less prominent First sign of sexual dimorphism in hemipenes size only ³	37–38	22.5 \pm 0.18	5.8 \pm 0.12	8.3 \pm 0.14
8	Signs of sexual dimorphism in shape of hemipenes, male more developed Scales differentiated on body Differentiation of cephalic scales and scales on digits ⁵ Scales present on eyelids	39	24.5 \pm 0.87	6.6 \pm 0.09	10.5 \pm 0.22
9	Hemipenes no longer visible in female, only vent visible at birth Acquired pigmentation shown at birth	40	26 \pm 0.16	7.1 \pm 0.9	10.7 \pm 0.16

Comparison with the development table developed for *Lacerta vivipara* (Dufaure and Hubert, '61) is indicated.

¹Occurs in Dufaure and Hubert ('61) stage 31.

²Occurs in Dufaure and Hubert ('61) stage 35.

³Occurs in Dufaure and Hubert ('61) stage 36.

⁴Occurs in Dufaure and Hubert ('61) stage 37.

⁵Occurs in Dufaure and Hubert ('61) stage 40.

by the development of the cortex with a concomitant reduction in the medulla; in males the opposite occurs, the cortex is reduced while the medulla develops and the seminiferous tubules form (Figs. 5a and b, respectively). The differ-

entiation of the male gonad appears to proceed more rapidly than that of the ovary, with the seminiferous tubules already present by stage 2 (Fig. 5b). By stage 6, the seminiferous tubules are separated by a significant amount of interstitial

	Lowland populations	Highland populations
January	8 ₉ Birth	6 ₇ 8 ₉ Birth
February		
March		
April		
May		
June	Hibernation	Hibernation
July		
August		
September	Ovulation	
October		Ovulation
November	1 2 3 4	1 2 3 4
December	5 6	5 4

Fig. 2. Diagrammatic representation of major events in the female reproductive cycle of *Niveoscincus ocellatus* with the stages of embryo development indicated (including representation of geographic, seasonal and inter-female variation (see Wapstra et al., '99).

tissue. The testis continues to develop, but with relatively few changes apparent in overall structure. The concurrent degradation in the medulla of the ovaries continues throughout embryonic development up to and including the final newborn stage with germ cells present in the cortex but follicle development absent (Fig. 5i).

In addition to the examination of changes in gonad structure during development, we further examined the development of the male and female reproductive ducts during development. The male Wolffian reproductive duct was apparent at the earliest stage identified here (Stage 1), while the gonad was still indifferent (Fig. 4). The Müllerian duct, however, is not present until stage 2. It is formed in both sexes, though it is

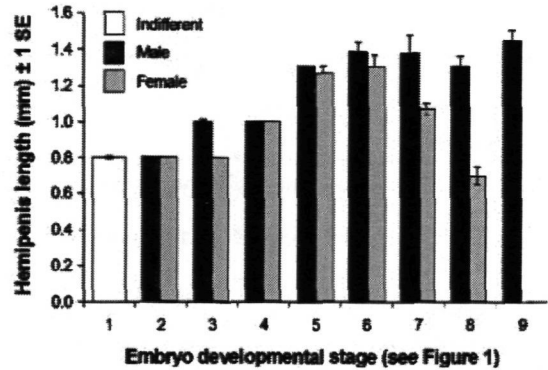


Fig. 3. Hemipenis development in male and female *Niveoscincus ocellatus* embryos. At stage 9, hemipenes in female embryos have largely regressed with the remaining gland retracted internally while male hemipenes remain external. At birth (stage 10), hemipenes are not visible in either males or females, although it is possible to evert hemipenes in males but not females in live offspring.

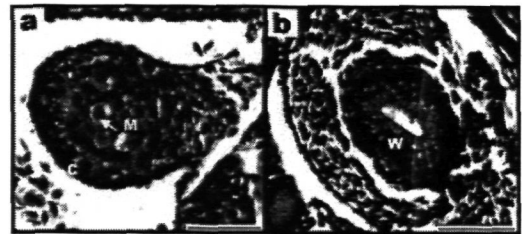


Fig. 4. The undifferentiated gonad (a) and associated reproductive ducts (b) of *Niveoscincus ocellatus* at stage 1. The female Müllerian duct has not yet formed and the male Wolffian duct is still associated with the mesonephric kidney. Bar = 50 μ m. C = cortex region, M = medulla region, W = Wolffian duct (archinephric duct).

substantially reduced in males compared with females (Figs. 6a and b). The duct continues to regress in males until it is lost altogether (stage 6; Fig. 6h). In females, this duct thickens and continues to develop until stage 6 (Fig. 6e), after which very little change is noted. Unlike the males, females retain the reproductive ducts of both sexes until after birth (stage 10; Fig. 6h).

DISCUSSION

Skinks are one of the largest families of squamates worldwide with their center of diversity in Australia (Cogger, 2000). The effects of incubation conditions (in oviparous species) and gestation conditions (in viviparous species) on offspring phenotype (e.g., Shine and Harlow, '96; Wapstra, 2000) including offspring sex (Shine

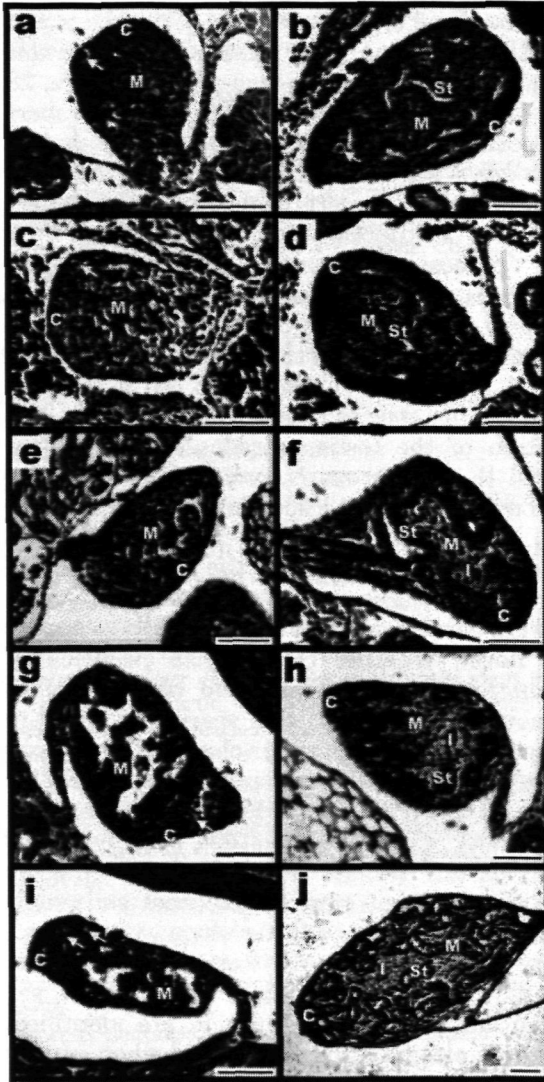


Fig. 5. Gonad development in female (a, c, e, g, i) and male (b, d, f, h, j) *Niveoscincus ocellatus*. (a and b): Stage 2; (c and d): stage 4; (e and f): stage 6; (g and h): stage 8; (i and j): stage 10. Bar = 50 μ m. St = seminiferous tubules, C = cortex region, M = medulla region, I = interstitial tissue, arrows indicate germ cells.

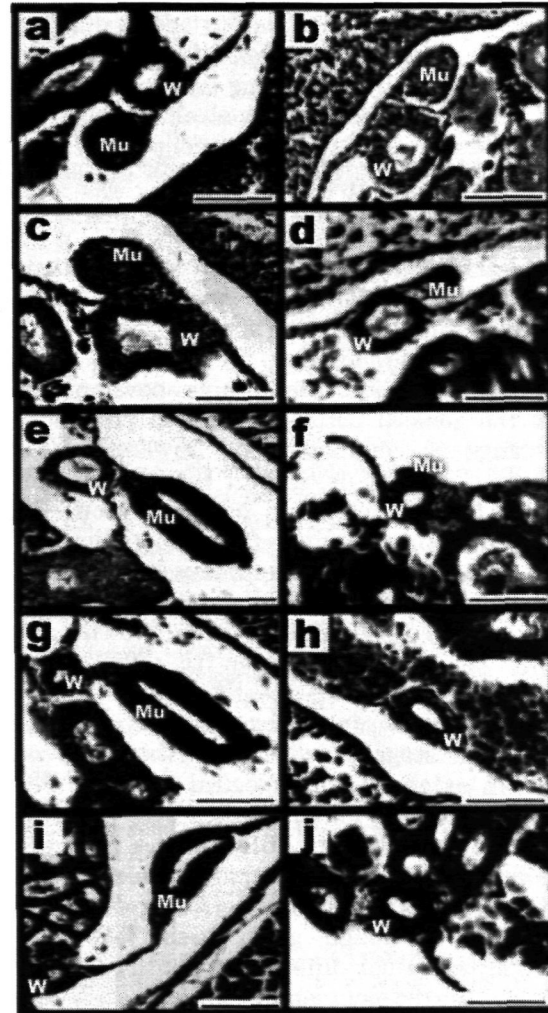


Fig. 6. Development of the reproductive ducts in female (a, c, e, g, i) and male (b, d, f, h, j) *Niveoscincus ocellatus*. (a and b): Stage 2; (c and d): stage 4; (e and f): stage 6; (g and h): stage 8; (i and j): stage 10, newborn. Bar = 100 μ m. Mu = Müllerian duct, female reproductive duct. W = Wolffian duct, the male reproductive duct.

et al., 1995; Robert and Thompson, 2001; Shine et al., 2002; Wapstra et al., 2004) are well studied. In fact, the taxon has become a model for the understanding of both sex determination (Shine, 1999) and the evolution of viviparity (e.g., Shine, 1995; Blackburn, 2000; Thompson et al., 2002 and references therein) in squamates because of the diversity of reproductive modes and modes of sex determination skinks possess. Despite this, there have been few if any studies that have examined

embryo development in skinks, either in relation to external morphological features or gonad development. Further interest in Australian skinks has been generated recently because two, only distantly related, species of viviparous skinks have been found to have TSD (Robert and Thompson, 2001; Wapstra et al., 2003, 2004). TSD and viviparity were previously thought to be mutually exclusive (Bull, 1980, and more recently Uller, 2003), and consequently studies on sex determination and differentiation were concentrated on oviparous species for understanding.

This paper has gone part way to addressing the lack of description of gonadal differentiation in viviparous skinks by examining embryonic development, including both external characters and gonad differentiation, in a viviparous Australian skink, *N. ocellatus*, a species that has the added attraction of possessing temperature effects on sex determination.

N. ocellatus reproduces annually throughout its range with a relatively long weather-dependent gestation; ovulation occurs in mid spring, with birth in mid-late summer (Jones et al., '97; Wapstra et al., '99). Embryonic development follows the general pattern described previously for a range of squamate taxa. Development is somewhat slow initially, in part because weather conditions are cooler early in the season. We concentrated our sampling efforts approximately 3–4 weeks into gestation (approximately 25% of total gestation time) but well before embryos were expected to begin significant body growth and certainly before gonads began to differentiate (Pieau et al., '99). We identified nine clearly identifiable stages in embryonic development (with a 10th stage at or near birth) based on changes in external morphological features. The only previous tables for squamates were provided by Dufaure and Hubert ('61) for the northern hemisphere lacertid, *L. vivipara*. This species exhibits broad similarities to *N. ocellatus* in development, although given their taxonomic separation the minor differences identified in the timing of some developmental characteristics were not unexpected. Of particular interest was the earlier development of hemipenes, and the earlier sexual differentiation in hemipene size, in *N. ocellatus*. As in *L. vivipara*, proto-hemipenes develop in both sexes and do not completely regress in females until very late in development. At birth, sexing of newborn *N. ocellatus* is relatively straightforward, with obvious differences in male and female organs (Wapstra et al., 2004, and see also Olsson and Shine, 2001); phenotypic sex as determined by hemipenis eversion was confirmed by gonad histology in stage-10 newborns in the present study.

Through a more rigorous sacrificing of females housed under standard conditions in the laboratory, Dufaure and Hubert ('61) were able to provide more stages (40). We feel, however that the nine stages we have identified are particularly useful for experimenters who are limited by ethical concerns and/or experimental limitations in the number of females that can be used to

assess the stage of development (obviously, working with viviparous taxa poses more significant difficulties than oviparous taxa). Furthermore, 28 of the 40 stages identified by Dufaure and Hubert ('61) occur before stage 1, and clearly before gonads have begun to differentiate and external factors (such as temperature) are likely to affect the process of differentiation/determination.

The process of gonad differentiation in *N. ocellatus* appears to follow the general pattern previously described for other reptilian species. The timing of differentiation occurs in the middle third of embryonic development, as it does in other reptilian species (Pieau, '96). Similarly, the formation of the testis precedes ovary development in the viviparous *N. ocellatus* as has been described in oviparous species (Merchant-Larios et al., '97; Ganesh et al., '99; Hewavisenthi and Parmenter, 2002). However, the early formation of interstitial tissue in the testis and the corresponding development of the hemipenes appear to be novel. Whether this reflects evolutionary differences between skinks and other reptiles, or a novel adaptation in this species will require further investigation of other closely related taxa.

To summarize, we have provided a workable developmental table that is likely to be adaptable for a variety of squamate reptiles, both oviparous and viviparous species. This study represents the first such study to examine external embryonic features concomitant with development of reproductive organs. This study is expected to become important as more unexpected influences on sex differentiation and determination are identified. This study also identifies the period when gonads are differentiating and potentially when the gonads are active (particularly males). This is crucial in designing further investigations where external influences on sex determination, such as the female hormone environment or female body temperature and behavior, are being investigated.

ACKNOWLEDGMENTS

This work was carried out with approval of animal ethics from M.U. (2001/011, 2003/009) and the University of Tasmania (No. 94070). This work was supported by grants to E.W. from the Australian Research Council (DP0211364), Macquarie University New Staff Grants Scheme and Macquarie University Grants Scheme. Lizards were caught under permit from Tasmanian Department of Primary Industries, Water and the Environment (Nature Conservation Branch,

permit numbers 95109, 95363, 96190, 02189, 01215, 01191 and 03159).

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