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In vitro progesterone production by maternal and embryonic tissues during gestation in the southern snow skink (*Niveoscincus microlepidotus*)

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Abstract

The southern snow skink, *Niveoscincus microlepidotus*, has a protracted gestation, lasting approximately one year. Ovulation occurs in spring (November) and embryonic development is completed by early autumn (March); however, birth does not occur until the following spring. Previous studies have shown that plasma progesterone concentration peaks in preovulatory females (spring, October), remains high during early gestation, and decreases to basal by autumn. In vitro progesterone production by corpora lutea, non-luteal ovary, anterior oviduct, placental tissues, muscle, and embryonic adrenal-gonads from *N. microlepidotus* was assessed throughout gestation. Tissues were incubated with or without the precursor pregnenolone for 3 h at 24 °C; the resulting media were analysed for progesterone using radioimmunoassay. In vitro progesterone production by corpora lutea in media only was high during early gestation, dropping to basal by autumn. Maternal adrenal glands produced progesterone in vitro in media only throughout gestation; however, the pattern of production did not correlate with plasma concentrations and may represent steroid that is normally converted to corticosterone. Non-luteal ovary, anterior oviduct, placental tissues, muscle, and embryonic adrenal-gonads produced minimal progesterone in media only, but were able to convert pregnenolone to progesterone; this suggests steroid metabolic capability within these tissues. Further research is needed to address the possible endocrine role(s) of placental and embryonic tissues during gestation in viviparous squamates.

Keywords: Adrenals; Corpora lutea; Gestation; Lizard; Placenta; Pregnancy; Progesterone; *Niveoscincus microlepidotus*; Viviparity

1. Introduction

Patterns of plasma progesterone concentrations associated with gestation vary widely among species of viviparous squamate (Jones and Baxter, 1991), with peaks occurring from periovulation (Girling et al., 2002), through mid (Bonnet et al., 2001; Edwards and Jones, 2001; Flemming, 1994; Kleis-San Francisco and Callard, 1986) to late gestation (Jones and Swain, 1996; Jones et al., 1997). In the red-sided garter snake (*Thamnophis sirtalis parietalis*), progesterone concentrations remained low or non-detectable throughout gestation (Whittier et al., 1987). The diversity of progesterone patterns amongst viviparous squamates reflects their diverse evolutionary history. Viviparity is believed to have evolved independently around 100 times in the squamate reptiles (Blackburn, 1982, 1985; Shine, 1985); it cannot be assumed that the mechanisms that maintain gestation are the same in each case.

The corpora lutea are believed to be the major source of progesterone in viviparous squamates (Callard et al., 1992; Xavier, 1987) and in the few species examined, patterns of plasma progesterone usually correlate with the activity of the corpora lutea (Bennett and Jones, 2002; Fergusson and Bradshaw, 1991; Flemming, 1994). In some species, however, plasma progesterone concentrations remain high even after regression of the corpora lutea (*Sceloporus jarrovi*, Guillette et al., 1981;

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Chalcides ocellatus and *Chalcides chalcides*, Angelini and Ghiara, 1991); therefore, an alternative source of this hormone must be postulated. Guillette et al. (1981) hypothesised that in *S. jarrovi* production of progesterone by corpora lutea may be replaced by production from corpora atretica or by placental tissues. In the viviparous skink *C. chalcides*, progesterone is produced in vitro by the placental tissues during the later stages of gestation (Guarino et al., 1998). It has also been hypothesised that adrenal glands may contribute to progesterone production during gestation (Bourne et al., 1986; Dauphin-Villemant and Xavier, 1985). However, a comprehensive analysis of progesterone production by different tissues over the course of gestation has not previously been undertaken in a viviparous squamate.

The southern snow skink, *Niveoscincus microlepidotus*, is a viviparous lizard found in alpine regions (above 1000 m elevation) of southern Tasmania, Australia (Hutchinson et al., 1989; Melville and Swain, 1999). These animals have an unusual biennial reproductive cycle that includes a protracted gestation period of approximately one year (Girling et al., 2002; Olsson and Shine, 1998, 1999; Rawlinson, 1974; Swain, 1972). Ovulation occurs in the austral spring (November) and embryonic development is completed by early autumn (March), approximately one month prior to hibernation. Parturition, however, does not occur until the following spring (November), several weeks after animals emerge from the winter hibernation period. Although the extended nature of this reproductive cycle is attributed to the cool and variable alpine habitat experienced by these animals (Hutchinson et al., 1989; Olsson and Shine, 1998), the physiological mechanisms by which gestation is extended are unknown. We initially hypothesised that plasma progesterone concentrations would remain high until after hibernation in *N. microlepidotus*; however, although concentrations were high ($15.4 \pm 5.9\,\mathrm{ng\,ml^{-1}}$) during the early stages of gestation (summer), they decreased to basal ($1.2 \pm 2.0\,\mathrm{ng\,ml^{-1}}$) by early autumn (March), prior to hibernation, and several months before parturition in late spring (Girling et al., 2002). It appears, therefore, that elevated plasma progesterone concentrations are not necessary for the maintenance of this long gestation period.

In this experiment, we measure in vitro progesterone production by maternal and embryonic tissues (adrenal glands, corpora lutea, non-luteal ovary, oviduct, placental tissues, muscle, and embryonic adrenal/gonads) over the course of gestation in *N. microlepidotus*. We hypothesise that corpora lutea will show the greatest capacity for progesterone production during gestation. Secondly, we consider alternative sources of progesterone; we investigate whether localised progesterone production by, perhaps, placental tissues continues after plasma concentrations decrease in autumn. Based on the very limited information available from other reptiles, we hypothesise that localised production by placental tissues may occur.

2. Methods and materials

2.1. Animal collection

Female *N. microlepidotus* in the following reproductive conditions were collected from January to November, 2000 ($n = 2$–6, see figures for details of samples sizes for each tissue): postovulation (November), early pregnancy (January), mid pregnancy (February), late pregnancy prior to winter (March), hibernation (June and August), late pregnancy after winter (October), and postpartum (November). Skinks were captured from the summit (1270 m) of Mt Wellington, Hobart, Tasmania, Australia (43° 51′S, 147° 18′E) and returned to the laboratory before dissection, usually the following day. These animals hibernate in deep rocky crevices over the winter and are inaccessible during this time. Animals needed for experiments over the winter were therefore collected in late autumn (March 2000) and returned to the laboratory. These skinks were provided with ample cover and housed in the refrigerator (4 °C) to mimic hibernation conditions. Water was provided ad libitum, but no food was provided. All procedures were approved by the University of Tasmania Animal Ethics Committee (A5676).

2.2. Dissection and incubation procedure

Prior to dissection, females were weighed and the snout-vent length (SVL) recorded. Animals were chilled to 4 °C for at least 30 min prior to decapitation. Tissues dissected from the females were the ovaries, adrenal glands, oviducts and muscle (from a hind limb). The corpora lutea were carefully dissected from the ovarian stroma and these tissues were incubated separately. As the different regions of the reptilian oviduct (the term 'oviduct' refers to the entire reproductive tract in reptiles) have different functions (Girling, 2002) and potentially different steroidogenic activity, we separated the oviduct into the anterior oviduct (infundibulum and uterine tube) and uterus. Then, the ovulated egg or embryo and associated extraembryonic membranes were gently pulled from the uterus. The extraembryonic membranes were then teased from the embryo. The uterus and extraembryonic membranes form the maternal and embryonic components of the placenta, respectively, and were incubated separately. Extraembryonic membranes were washed in incubation medium to remove any remaining yolk prior to incubation. Postovulation (November), the extraembryonic membranes were not incubated, as they could not be removed from the newly ovulated egg. In early pregnancy (January), the extraembryonic membranes could be further separated into the yolk sac

membranes and the chorioallantoic membranes and thus these were incubated separated. Once embryos reached a size at which dissection was possible (February–October), their gonads (with associated adrenal glands) were removed and incubated. Hind limb muscle was included as a control tissue that was not expected to produce progesterone in vitro.

All tissues were incubated in 500 μl of incubation medium (L-15 Medium Leibovitz, Sigma Chemicals, St. Louis, USA) for a period of 3 h at 24 °C. This temperature was chosen as it approximates the preferred body temperature of pregnant *N. microlepidotus* (Kabat, 1999). The tissues from individual females ($n = 2$–5 per month, see figures for all sample sizes) were incubated either with or without excess pregnenolone precursor (5 μg in ethanol, air-dried to evaporate) to assess basal versus maximal production rates. At the completion of the incubation period, incubation medium was decanted into clean test-tubes and frozen at −20 °C until analysis for progesterone using radioimmunoassay. Cross-reactivity of pregnenolone in the assay was quantified (see below). Incubated tissues were dried and weighed to allow calculation of progesterone production per milligram of tissue. The small size of corpora lutea, adrenal glands, and embryonic adrenal-gonads prevented an accurate mass being obtained. Hence, in these cases, progesterone production was expressed per corpus luteum, adrenal, or adrenal-gonad.

2.3. Progesterone radioimmunoassay

Aliquots of incubation medium (200 μl) were extracted once with 3 ml iso-octane (2,2,4-trimethyl-pentane) and the extract analysed by radioimmunoassay. Extraction efficiency, determined from recovery of label from spiked media, was greater than 90% (coefficient of variation ranging from 5–7%). Extracted samples were incubated overnight at 4 °C with [1,2,6,7,16,17-^{3}H]Progesterone (TRK 641, Amersham Pharmacia Biotech, Sydney, Australia) and progesterone antiserum (P11-192, Endocrine Sciences, CA, USA; cross reactivities: Deoxycorticosterone 3.3%, 4-pregnen-20β-ol-3-one 1.3%, 20 other steroids <1%). Samples were assayed after bound and unbound fractions were separated with a dextran–charcoal solution. The minimum detectable value on the standard curve was 6.25 pg. Media spiked with progesterone ran parallel to the standard curve. The interassay and intraassay coefficients of variation were 14.2% ($n = 12$) and 14.3% ($n = 9$), respectively. Progesterone was non-detectable in media only. Potential cross-reactivity of the precursor pregnenolone with the progesterone antibody was checked by assaying media containing pregnenolone ($n = 10$ tubes); cross-reactivity was equivalent to 46 ± 4.2 pg progesterone/incubation tube (means ± SEM). Thus, 46 pg was removed from the total quantity of progesterone detected in tubes containing tissues incubated with pregnenolone, prior to data analysis and display.

2.4. Statistics

Statistical analyses were carried out using Systat (version 5.2, SPSS, Chicago, USA). All means are reported ± one standard error. A p-value $\leqslant 0.05$ was considered to be significant. To conform to the requirements of ANOVA (normality and homoscedasticity), all data were log-transformed prior to analysis.

In vitro progesterone production by corpora lutea and adrenal glands was analysed using two-way ANOVAs, followed by Tukey posthoc tests. For the ovarian stroma, anterior oviduct, uterus, extraembryonic membranes, embryonic adrenal-gonads, and muscle, in vitro progesterone production was below or close to the minimum detectable concentration when incubated in media only; thus, only results from experiments of tissues incubated with pregnenolone were analysed statistically. A one-way ANOVA, followed by Tukey posthoc tests, was used. Due to the low sample sizes in some months, data concerning progesterone production by adrenal glands in January and February, and August and October, and by the anterior oviduct in June and August, have been combined for statistical analysis (but separated for display). In February, extraembryonic membranes could be separated into chorioallantoic and yolk sac membranes and in vitro progesterone production by these two tissue compartments was compared using a t test. Production data from the two membranes were summed prior to inclusion in the ANOVA.

3. Results

3.1. Corpora lutea

In vitro progesterone production by corpora lutea varied significantly with month ($F_{(7,45)} = 26.9, p = 0.001$) and in the presence or absence of pregnenolone ($F_{(1,45)} = 442.6, p = 0.001$); there was a significant interaction effect ($F_{(7,45)} = 12.3, p = 0.001$). In vitro progesterone production by corpora lutea incubated in media only peaked in early gestation (January: 3.7 ± 0.29 ng CL^{-1}), decreasing significantly by mid-gestation ($p = 0.001$, February: 0.5 ± 0.16 ng CL^{-1}) (Fig. 1A). Production in media only was basal prior to hibernation when females carried fully developed embryos (March: 0.03 ± 0.006 ng CL^{-1}); however, there was a small increase in production during early winter (June: 0.3 ± 0.34 ng CL^{-1}), returning to basal by the end of winter.

In vitro progesterone production by corpora lutea in the presence of pregnenolone remained high throughout gestation, with no significant variation among months (Fig. 1B).

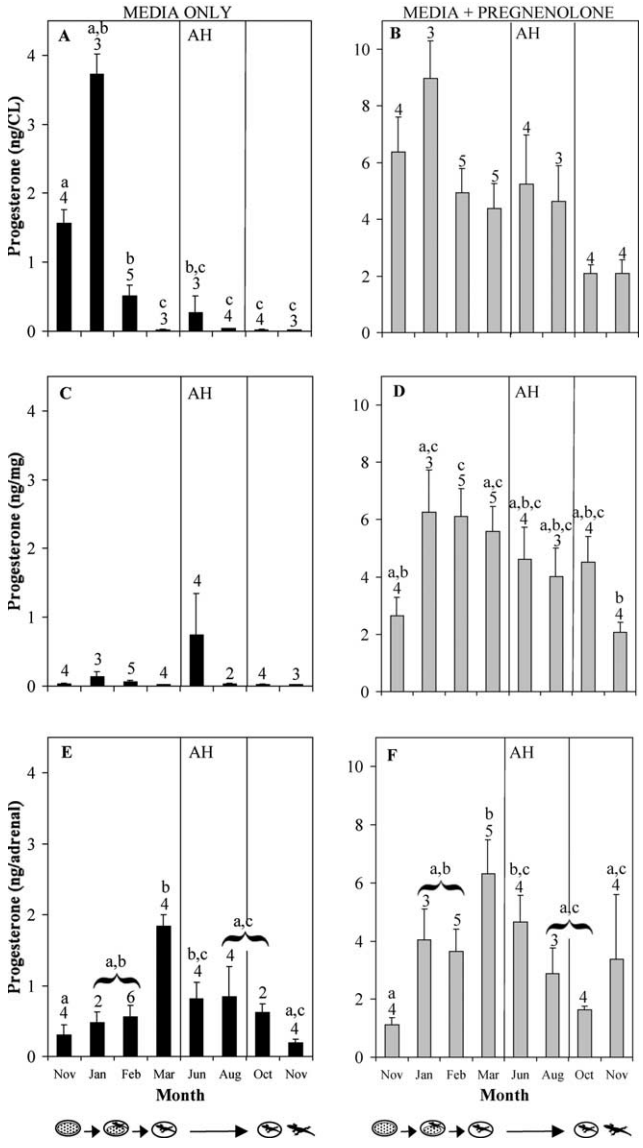

Fig. 1. Mean in vitro progesterone production by tissues from the southern snow skink, *Niveoscincus microlepidotus*, at different stages of gestation. Corpora lutea (A) in media only or (B) with excess pregnenolone, non-luteal ovary (C) in media only or (D) with excess pregnenolone, and adrenal glands (E) in media only or (F) with excess pregnenolone. Please note the difference in scales between each set of graphs. Means illustrated between the two vertical lines were determined from females maintained under artificial hibernation (AH) conditions. Error bars represent one standard error of the mean. Numbers above the error bars are sample sizes. Low case letters above the error bars represent months that are significantly different from each other ($p < 0.05$). Months bracketed were combined for statistical analysis. Diagrams at the base of the figure represent the stage of embryonic development in utero during pregnancy, respectively (from postovulation to postpartum).

3.2. Ovarian stroma

Little or no in vitro progesterone production occurred when ovarian stroma was incubated in media only (Fig. 1C). The small peak in production observed in early winter (June: $0.7 \pm 0.61\,\text{ng mg}^{-1}$) consisted of one sample of $2.6\,\text{ng mg}^{-1}$ and three others less than $0.4\,\text{ng mg}^{-1}$. In vitro progesterone production in the presence of pregnenolone changed significantly during gestation ($F_{(7,24)} = 4.1,\ p = 0.005$) (Fig. 1D). Production was high throughout most of gestation; production by postovulatory females ($2.6 \pm 0.66\,\text{ng mg}^{-1}$) was significantly less than that in pregnant females in February ($p = 0.05$, $6.4 \pm 1.01\,\text{ng mg}^{-1}$), and production by postpartum females (November: $2.2 \pm 0.35\,\text{ng mg}^{-1}$) was significantly less than that in pregnant females in January, February, and March ($p = 0.026$, 0.010, and 0.028, respectively; 6.6 ± 1.8, 6.4 ± 1.01 and $5.7 \pm 0.87\,\text{ng mg}^{-1}$, respectively).

3.3. Maternal adrenal glands

In vitro progesterone production by adrenal glands was detectable in all months, even when incubated in media only. In vitro progesterone production by maternal adrenal glands varied significantly with month ($F_{(5,49)} = 7.0, p = 0.001$) and was significantly higher in the presence of pregnenolone ($F_{(1,49)} = 68.4, p = 0.001$); there was no interaction effect (Fig. 1E, F). In vitro progesterone production by adrenal glands from females with fully developed embryos prior to hibernation (March: media only: $1.8 \pm 0.16\,\text{ng adrenal}^{-1}$, media plus pregnenolone: $7.2 \pm 0.90\,\text{ng adrenal}^{-1}$) was significantly higher than production in postovulatory females (November: $p < 0.001$, media only: $0.3 \pm 0.14\,\text{ng adrenal}^{-1}$ and media + pregnenolone: $1.3 \pm 0.25\,\text{ng adrenal}^{-1}$), females in late gestation at the end of hibernation (August/October: $p = 0.005$, media only: $0.8 \pm 0.23\,\text{ng adrenal}^{-1}$, media + pregnenolone: $2.4 \pm 0.65\,\text{ng adrenal}^{-1}$), and postpartum females (November: $p = 0.001$, media only: $0.2 \pm 0.05\,\text{ng adrenal}^{-1}$, media + pregnenolone: $3.5 \pm 2.23\,\text{ng adrenal}^{-1}$).

3.4. Anterior oviduct

Anterior oviductal tissues produced little or no progesterone in vitro when incubated in media only (Fig. 2A). Progesterone production did occur, however, if pregnenolone was provided (Fig. 2B). Production in the presence of pregnenolone changed significantly during gestation ($F_{(6,23)} = 4.4, p = 0.004$). Although there were no significant differences in progesterone production between some stages of gestation, production in early gestation (January: $2.6 \pm 0.41\,\text{ng mg}^{-1}$) and prior to hibernation (March: $2.6 \pm 0.49\,\text{ng mg}^{-1}$) was significantly higher than production in late gestation after hibernation (October: $p = 0.048$ and 0.045, respectively; $0.8 \pm 0.22\,\text{ng mg}^{-1}$) and post parturition (November: $p = 0.016$ and 0.013, respectively; $0.6 \pm 0.11\,\text{ng mg}^{-1}$).

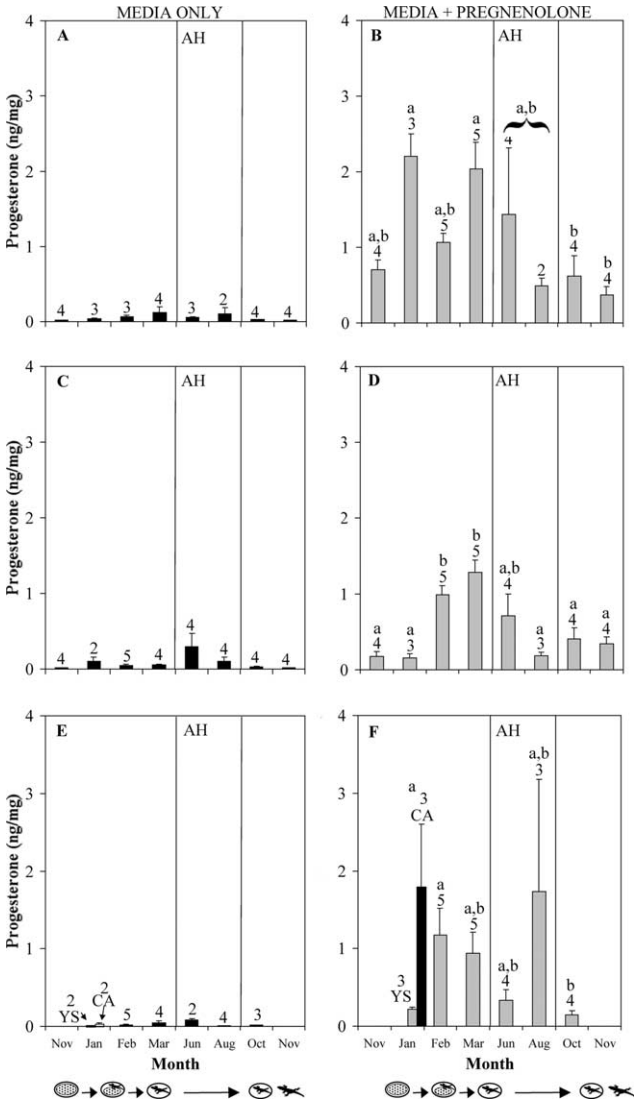

Fig. 2. Mean in vitro progesterone production by tissues from the southern snow skink, *Niveoscincus microlepidotus*, at different stages of gestation. Anterior oviduct (A) in media only or (B) with excess pregnenolone, uterus (C) in media only or (D) with excess pregnenolone, and extraembryonic membranes (E) in media only or (F) with excess pregnenolone. Please note the difference in scales between each set of graphs. Extraembryonic membranes could not be removed from the newly ovulated egg in November and were, therefore, not incubated. In January, extraembryonic membranes could be separated into the yolk sac (YS) and chorioallantoic (CA) membranes. Means illustrated between the two vertical lines were determined from females maintained under artificial hibernation (AH) conditions. Error bars represent one standard error of the mean. Numbers above the error bars are sample sizes. Low case letters above the error bars represent months that are significantly different from each other ($p < 0.05$). Months bracketed were combined for statistical analysis. Diagrams at the base of the figure represent the stage of embryonic development in utero during pregnancy, respectively (from postovulation to postpartum).

3.5. Placenta

3.5.1. Uterus

Uterine tissues produced little or no in vitro progesterone when incubated in media only, except for a small peak in June during artificial hibernation (0.3 ± 0.17 ng mg^{-1}; Fig. 2C). Progesterone production by uterine tissues in the presence of pregnenolone changed significantly during gestation ($F_{(7,23)} = 10.3, p = 0.001$; Fig. 2D); production was generally low but detectable. Progesterone production in mid gestation prior to hibernation (February: 1.2 ± 0.15 ng mg^{-1}; March: 1.5 ± 0.17 ng mg^{-1}) was significantly higher than production in the early stages of gestation (November: $p = 0.003$ and 0.001, respectively; 0.3 ± 0.06 ng mg^{-1}; January: $p = 0.004$ and 0.002, respectively; 0.2 ± 0.07 ng mg^{-1}) or in late gestation in late winter (August: $p = 0.021$ and 0.008, respectively; 0.3 ± 0.05 ng mg^{-1}).

3.5.2. Extraembryonic membranes

Extraembryonic membranes produced little or no progesterone in vitro when incubated in media only (Fig. 2E). In vitro progesterone production in the presence of pregnenolone changed significantly during gestation ($F_{(5,17)} = 3.8, p = 0.018$). Production in early-mid gestation prior to hibernation (January: 1.1 ± 0.88 ng mg^{-1}; February: 1.4 ± 0.40 ng mg^{-1}) was significantly greater than in late gestation after hibernation (October: $p = 0.026$ and 0.029, respectively, 0.2 ± 0.05 ng mg^{-1}). Mean production in August consisted of one tissue sample producing 4.0 ng mg^{-1}, while two other samples produced less than 0.6 ng mg^{-1}. Extraembryonic membranes collected in January were separated into the yolk sac and chorioallantoic membranes. Chorioallantoic membranes may produce more progesterone than yolk sac membranes, although there was no significant difference at the 95% confidence level ($t_{(4)} = 2.2, p = 0.09$).

3.6. Embryonic adrenal-gonads

In vitro progesterone production by embryonic adrenal-gonads was generally low when incubated in media only (Fig. 3A). In vitro progesterone production in the presence of pregnenolone changed significantly over the course of gestation ($F_{(4,16)} = 4.8, p = 0.01$; Fig. 3B). Production in March (6.4 ± 2.3 ng adrenal-gonad^{-1}) was significantly higher than production in mid gestation (February: $p = 0.011$, 1.8 ± 0.29 ng adrenal-gonad^{-1}) and late gestation after hibernation (October: $p = 0.019$, 1.6 ± 0.26 ng adrenal-gonad^{-1}). Amounts of progesterone produced were similar to those produced by maternal adrenal glands, despite the much smaller size of the embryonic adrenal-gonads.

3.7. Muscle

Muscle produced little or no progesterone when incubated in vitro in media only (Fig. 4A). In the presence of pregnenolone, production changed significantly over the course of gestation ($F_{(6,22)} = 15.4, p = 0.001$). Production in the presence of pregnenolone remained low throughout

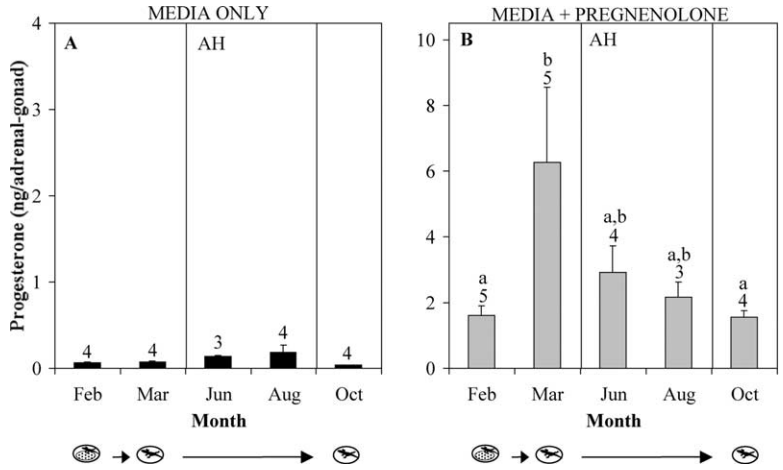

Fig. 3. Mean in vitro progesterone production by adrenal-gonads from embryonic southern snow skinks, *Niveoscincus microlepidotus*, at different stages of gestation. Embryonic adrenal-gonads were incubated (A) in media only or (B) with excess pregnenolone. Please note the difference in scales between each set of graphs. Means illustrated between the two vertical lines were determined from embryos dissected from females maintained under artificial hibernation (AH) conditions. Error bars represent one standard error of the mean. Numbers above the error bars are sample sizes. Low case letters above the error bars represent months that are significantly different from each other ($p < 0.05$). Diagrams at the base of the figure represent the stage of embryonic development in utero during pregnancy.

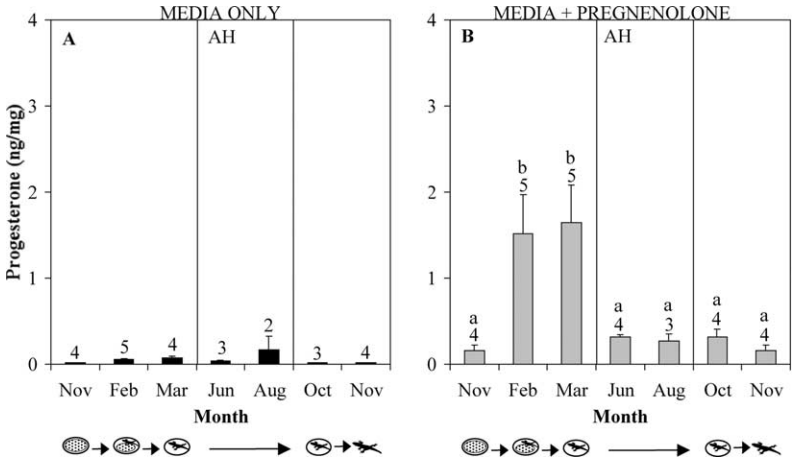

Fig. 4. Mean in vitro progesterone production by muscle from female southern snow skinks, *Niveoscincus microlepidotus*, at different stages of gestation. Muscle was incubated (A) in media only or (B) with excess pregnenolone. No muscle samples were incubated in January. Means illustrated between the two vertical lines were determined from females maintained under artificial hibernation (AH) conditions. Error bars represent one standard error of the mean. Numbers above the error bars are sample sizes. Low case letters above the error bars represent months that are significantly different from each other ($p < 0.05$). Diagrams at the base of the figure represent the stage of embryonic development in utero during pregnancy.

gestation, except for a significant increase (p values all <0.005) in production during mid to late gestation prior to hibernation (February: $1.9 \pm 0.51\,\mathrm{ng\,mg^{-1}}$; March: $1.8 \pm 0.44\,\mathrm{ng\,mg^{-1}}$, respectively; Fig. 4B).

4. Discussion

Various in vitro incubation experiments have indicated that corpora lutea of reptilian species are able to produce progesterone (for example: Ciarcia et al., 1993; Gobbetti et al., 1993; Xavier, 1982); activity of the enzyme 3β-hydoxy-Δ^5-steroid dehydrogenase (3β-HSD), responsible for conversion of pregnenolone to progesterone, has also been reported (for example: Guarino et al., 1998). Results from our incubation experiments with tissues from the southern snow skink, *N. microlepidotus*, were consistent with the above observations and supported our first hypothesis that, compared with other tissues studied, the corpora lutea would show the greatest capacity for progesterone production during gestation. In vitro production of progesterone by corpora lutea in media only was high in females in the early stages of gestation (November–January), decreased to

basal by autumn (March) and remained low during the remainder of gestation. The in vitro progesterone production by corpora lutea in *N. microlepidotus* mirrored plasma progesterone concentrations during gestation (i.e., high in early gestation, decreasing to basal prior to hibernation) (Girling et al., 2002). However, even after plasma levels had dropped, and production in media only had decreased, our results clearly show that corpora lutea retain the capacity to produce progesterone if pregnenolone is provided. This suggests that progesterone production by corpora lutea is limited by the availability of steroid precursors. Similarly, the capacity of non-luteal ovarian tissue to produce progesterone during gestation when supplied with the precursor pregnenolone reflects the potential steroidogenic capacity of this tissue.

It has previously been hypothesised that the adrenal glands may contribute to plasma progesterone concentrations in viviparous squamates (Bonnet et al., 2001; Bourne et al., 1986; Highfield and Mead, 1975a, 1975b). In the viviparous snake *Vipera aspis*, plasma progesterone concentrations in males are as high as those in non-reproducing females (Bonnet et al., 2001). The authors suggest that this progesterone may be of adrenal origin. The adrenal glands from *N. microlepidotus* consistently produced progesterone in vitro throughout gestation, even without the precursor pregnenolone. However, in the close relative *Niveoscincus metallicus*, in vitro progesterone production by adrenal glands was only one tenth (approximately) that of the corpora lutea (Bennett and Jones, 2002). In *N. microlepidotus*, the peak in in vitro progesterone production by adrenal glands occurred in autumn (March), when plasma progesterone concentrations are basal (Girling et al., 2002), but when plasma corticosterone concentrations are at their peak (C. de Mestre, S.M. Jones, and J.E. Girling, unpublished data). Thus, it is difficult to determine whether the progesterone produced by adrenal glands in vitro would normally be secreted or whether it represents an intermediate in the production of corticosterone, the major adrenal corticosteroid in reptiles. Of further interest was the peak in in vitro progesterone production by adrenal-gonads from embryonic *N. microlepidotus*, which also occurred in autumn (March, discussed below).

Although progesterone production by placental tissues may replace that of the corpora lutea in certain viviparous squamates, our incubation data do not support such a role for the placenta in *N. microlepidotus*, despite the very long gestation period exhibited by this species. In vitro production of progesterone in media only was low or non-detectable throughout gestation in *N. microlepidotus*; therefore, our second hypothesis of localised progesterone production after plasma concentrations had decreased was not supported. However, the in vitro progesterone production in the presence of pregnenolone by both maternal (uterus) and embryonic (extraembryonic membranes) components of the placenta, as well as the anterior regions of the oviduct, suggests that these tissues are capable of steroid metabolism and may be involved in localised production of steroids.

Progesterone production by placental tissues in viviparous squamates was initially suggested by Guillette et al. (1981). They found that plasma progesterone concentrations remained high during gestation in the viviparous lizard *S. jarrovi*, even after regression of the corpora lutea. They hypothesised that progesterone production by corpora lutea was replaced by either placental tissues or the corpora atretica (previtellogenic follicles develop during gestation in *S. jarrovi* and become atretic during late gestation to form corpora atretica). Interestingly, preliminary results suggest that placental tissues from *S. jarrovi* do not produce detectable amounts of progesterone, even when incubated in the presence of pregnenolone; these studies, however, are still ongoing (Painter and Moore, 1998). However, in the viviparous skink *C. chalcides*, in vitro progesterone production (in media only) by placental tissues was low during the early stages of gestation, but increased towards the later stages of gestation; the opposite pattern was observed for incubations of corpora lutea in that species (data based on sample sizes of two only; no indication of variation between samples was provided; Guarino et al., 1998). Histochemical reactions for 3β-HSD in placental tissues of *C. chalcides* were negative in early and mid-gestation, but intensely positive in the maternal component (uterus) of the placenta during late gestation. This is the only report in the literature in which placental steroidogenic capacity has been confirmed for a reptilian species. Our understanding of the complexity and diversity of gestation in viviparous squamates will be further advanced by examination of the steroid activity within their placental tissues and consideration of how this activity varies among different species that have evolved viviparity independently.

Unexpectedly, hind limb muscle that was provided with the pregnenolone precursor produced some progesterone during mid to late gestation prior to hibernation (February and March). Like all tissues, muscle contains steroidogenic enzymes and the incubation results may reflect a generalised increase in steroidogenic enzyme activity during the warmer months. Alternatively, there is some evidence of greater enzyme activity in muscle from gestating females in comparison to non-gestating females: in the blue tongued skink, *Tiliqua nigrolutea*, muscle from gestating females has a greater capacity to conjugate steroids than that of non-gestating females (Edwards, 1999).

During gestation in *N. microlepidotus*, both plasma progesterone (Girling et al., 2002) and in vitro progesterone production by corpora lutea decrease to basal by autumn, when embryonic development is completed,

but well before the time of parturition the following spring. Our results, therefore, do not support an alternative source of progesterone replacing or augmenting production by the corpora lutea. Small peaks (but with large variation around the mean) in progesterone production by corpora lutea, non-luteal ovary, and uterus were observed in early winter (June), but it was difficult to determine whether all or any of these tissues contribute to the winter peak in plasma progesterone concentrations. It should also be noted that all incubations were carried out at 24 °C, the preferred body temperature of pregnant *N. microlepidotus* (Kabat, 1999). Females in winter, however, including those hibernated in the laboratory, do not experience such high temperatures. We are currently investigating how variations in temperature affect steroid hormone production by different tissues.

Thus, there is no evidence to support our original hypothesis (Girling et al., 2002) that progesterone is responsible for maintaining the extended gestation period in *N. microlepidotus*. As mentioned above, the decrease in progesterone production by corpora lutea occurs around the same time that embryonic development is completed. In this regard, the pattern of plasma progesterone is consistent with that observed in two closely related species of skink, *Niveoscincus metallicus* (Jones and Swain, 1996) and *Niveoscincus ocellatus* (Jones et al., 1997). In these two species, which exhibit an annual reproductive cycle, plasma progesterone concentration is high during early gestation, decreasing as embryonic development is completed, just before parturition. These observations suggest that our question concerning the protracted nature of gestation in *N. microlepidotus* should be refocussed. Is it parturition that is being prevented, rather than an extension of gestation per se. If the question is to be addressed from this point of view, interactions between temperature, photoperiod, and components of the parturition process need to be investigated.

To our knowledge, no previous study has considered the steroidogenic activity of embryonic adrenal glands and gonads in a viviparous squamate. Although little or no progesterone was produced by *N. microlepidotus* embryonic adrenal-gonads when incubated in media only, the progesterone production in the presence of pregnenolone indicate the steroidogenic capacity of these tissues. This is not a surprising result; these tissues are presumably active during embryonic development and growth. The peak production capacity occurred in autumn (March), corresponding to the peak in production of progesterone by adult female adrenals and the peak in plasma corticosterone concentrations (C. de Mestre, S.M. Jones, and J.E. Girling, unpublished data). Does this reflect similar activity by embryos and the mother in preparation for hibernation, or does it reflect activity in the embryo as development and growth are completed? Could additional activity by adrenal-gonads in March represent a mechanism by which embryos prevent parturition occurring prior to winter? That the embryo is involved in controlling the timing of parturition is well known for a number of eutherian mammal species (Challis and Olsson, 1998) and the possibility of similar control should not be discounted in viviparous squamates.

There has been a tendency to provide simplistic explanations for the maintenance of gestation in reptiles; however, complex interactions between the embryo(s) and placental tissues, as seen in eutherian mammals, can not be excluded, particularly in those species exhibiting complex placentae and matrotrophy. Further research is required to determine how the squamate placentae and embryos interact to influence gestation and the timing of parturition. The temporal separation of the completion of embryonic development and parturition during the protracted gestation period of *N. microlepidotus* will continue to provide an ideal model system for such investigations.

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