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General and Comparative Endocrinology 144 (2005) 204–210
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Seasonal variations in reproductive hormones in free-ranging echidnas (*Tachyglossus aculeatus*): Interaction between reproduction and hibernation

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Received 22 March 2005; accepted 28 May 2005
Available online 28 July 2005

Abstract

We measured the reproductive steroids testosterone and progesterone in free-ranging adult echidnas over several years. For months other than June–August, the mean progesterone concentration was 0.18 ± 0.12 ng ml^{-1} ($n = 14$), and all blood samples taken from active female echidnas in June–August had progesterone concentrations above 0.5 ng ml^{-1}. The highest progesterone value measured was 13.4 ng ml^{-1} in a pregnant female several days before egg-laying. For months other than June–August the mean testosterone concentration was 0.09 ± 0.05 ng ml^{-1} ($n = 13$). During June–August all active sexually mature males had testosterone concentrations in excess of 0.2 ng ml^{-1} and were found in mating groups at some time during this period. The highest plasma testosterone concentration measured was 4.62 ng ml^{-1}.

Keywords: Monotreme; Echidna; Reproduction; Testosterone; Progesterone; Hibernation

1. Introduction

Given the unique reproductive status of the monotremes as egg-laying mammals, information on their reproductive physiology is remarkably patchy. Monotreme oviparity is not simply a plesiomorphic retention of a reptilian reproductive pattern, nor a variation on that of birds. Nutrients for the developing bird embryo come exclusively from the yolk, and yolk is the primary source of nutrients for embryonic reptiles (Swain and Jones, 2000). In monotremes, however, the contribution of the yolk is nearly negligible, and nourishment for embryonic growth and differentiation is overwhelmingly from the protein-rich secretions of the uterine glands (Hill, 1933, 1941). The yolk-sac of monotremes is shelled, unlike that of other mammals (Renfree and Shaw, 1999), but the shell is porous, and it is probable that uptake of nutritive fluid continues until egg-laying (Hill, 1933; Oftedal, 2002). Uterine secretions stored in the egg also provide the nutrition for growth during the incubation period (Hill, 1941). Thus, female monotremes have combined mammalian and reptilian features of reproduction by integrating a uterine luteal secretory phase into an otherwise fundamentally sauropsid-like mode of reproduction (Gemmell, 1995).

Consistent with its role in other mammals and in reptiles (Jones and Baxter, 1991), progesterone is likely to have an important role in preparing the monotreme uterus to receive the egg, particularly in pre-ovulatory maturation of the uterine glands, and in maintaining uterine secretions during gestation (Gemmell, 1995). Large increases in plasma and faecal progesterone have been observed in female platypuses during the breeding

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doi:10.1016/j.ygcen.2005.05.013

season (Jabukowski et al., 1998), but while field studies such as this provide information about reproductive patterns at the population level, there are no serial data from individual females through pregnancy, and there is no published information on progesterone in female echidnas. There is a similar paucity of published information on the hormonal control of reproduction in male echidnas (Temple-Smith and Grant, 2001), although large increases in plasma and faecal androgens have been observed before and during the breeding season in male platypuses (Handasyde et al., 1992; McFarlane and Carrick, 1992; New et al., 1998).

Both platypuses and echidnas have long been known to be seasonal breeders (Griffiths, 1978), but the reproductive cycle of echidnas is complicated by the fact that, in all echidna populations studied to date, reproduction appears to follow a reduction in activity, which may range from a few days of torpor on Kangaroo Island (Rismiller and McKelvey, 1996) to months of deep hibernation in the Australian Alps and Tasmania (Beard et al., 1992; Grigg et al., 1989; Nicol and Andersen, 1996, 2002). In an unpublished study, Dean (2000) demonstrated an annual cycle in both plasma and faecal steroids in free-ranging echidnas on Kangaroo Island. Plasma testosterone concentrations of males ranged from 1 to 5.7 ng ml^{-1}, with an annual peak in July. In females, plasma progesterone values ranged from 0.2 to 6.9 ng ml^{-1}, but no female sampled was known to have subsequently produced young. Faecal pregnanes, including progesterone, were measured on a larger number of animals, and showed a strong seasonal cycle, being raised from June to October, with a peak in July. There was no discernable seasonal change in faecal oestradiol, although there was a significant rise in oestrone in June–July. Aspects of this work were duplicated in two much less comprehensive studies of faecal steroid concentrations in captive echidnas (Oates et al., 2002; Higgins et al., 2004), but neither of these assessed plasma hormone concentrations.

Tasmanian echidnas (*Tachyglossus aculeatus setosus*) differ from the Kangaroo Island echidnas (*Tachyglossus aculeatus multiaculeatus*) studied by Dean (2000) not only in their physical appearance (Griffiths, 1989) but also in aspects of their reproductive behaviour, and in their use of hibernation. Kangaroo Island echidnas show only short periods of torpor and inactivity before the mating season and during the mating season they form mating trains of up to 7 males that follow a female for up to 5 weeks (Rismiller and McKelvey, 1996). In contrast, Tasmanian echidnas show a prolonged period of deep hibernation (Nicol and Andersen, 2002), the timing of which is closely linked to reproductive activity. Reproductively mature male Tasmanian echidnas enter hibernation around mid-February, with reproductively mature females entering hibernation about a month later (Nicol and Andersen, 2002). Reproductively mature females may arouse from hibernation from mid-June onwards, and mate soon afterwards, or may hibernate through until October and not mate that year (Nicol and Andersen, 2002). The majority of the males arouse from hibernation significantly earlier than the females; as early as mid-May (Nicol and Andersen, 2002). To date there is no published information on hormonal control of reproductive seasonality of echidnas that show deep hibernation.

The overall aim of the present study was thus to investigate the seasonal variations in the major reproductive steroids in free-ranging reproductively mature male and female Tasmanian echidnas, *Tachyglossus aculeatus setosus*. As well as providing this baseline information, we aimed to answer several specific questions. Are there major differences in testosterone levels between males during the mating season which may reflect their degree of involvement in breeding activity and which may be linked to breeding success? Are elevated plasma progesterone levels in females a reliable indicator of pregnancy? How soon after hibernation do changes in reproductive hormones occur? Is there a drop in progesterone preceding egg-laying?

2. Materials and methods

The study was carried out on a population of animals on a grazing property ("Lovely Banks") 50 km north of Hobart, Tasmania. Since 1996 we have tagged 149 adult echidnas (82 female, 67 males) in the approximately 12 km^2 study area. Echidnas were initially captured by hand and tagged with implantable microchips (Life Chip, Destron Fearing) and 18 (8 male, 10 female) animals had radio transmitters glued to the spines on the lower back to allow them to be relocated for repeated blood sampling.

2.1. Reproductive status

Animals that were known to have been involved in reproductive activity in previous years were selected for inclusion in the study. Some other animals were opportunistically sampled, however, the majority of these were found in mating groups, and as echidnas are normally solitary, aggregations of animals can be considered to be evidence of reproductive activity (Beard and Grigg, 2000). On the basis of intensive, virtually continuous, field observations of echidnas on Kangaroos Island, Rismiller and McKelvey (2000) described four stages of male courtship, ranging from of stage 1, with males close to the female, but with little physical contact, to stage 4, which ends in copulation. Tasmanian echidnas often mate in logs, or underground, when they cannot be observed. Because of this much lower likelihood of observing much of the courtship

behaviour we have simply recorded whether males were in a mating group or not, and if they were observed mating. For females, information on mating activity and gestational state is important for the interpretation of progesterone data. However, males may be attracted to non-breeding females (Rismiller and McKelvey, 2000) and thus finding a female with one or more males does not necessarily indicate she is reproductively active. Ranked in increasing order of certainty we have used the following indicators of pregnancy: found with males; mating; pouch development and swollen mammary glands; construction of a nursery burrow. While the presence of an egg or a young in the pouch provided unequivocal evidence of pregnancy, a further indicator of a successful pregnancy is available from animals with an implanted body temperature logger: females show a characteristic reduction in body temperature variability when they are in the nursery burrow (Beard and Grigg, 2000; Nicol and Andersen, 2002), due to the reduction in activity (Nicol et al., 2004).

2.2. Blood sampling

Blood samples were taken from the rostral sinus under halothane or isoflurane anaesthesia. The majority of samples were taken in the field within 1 h of capture, but 14 samples from males and 10 from females were taken from animals after they were brought into the laboratory, in which case they were sampled within 24 h of capture. Approximately 1 ml of blood was taken into a plastic syringe that was then placed on ice. Blood samples were centrifuged and plasma stored at −20 °C until analysis. Samples taken in 2000 were packed in dry ice and transported to Brisbane for testosterone analysis by Dr A. Bradley as described previously (Millis and Bradley, 2001).

2.3. Radioimmunoassay of testosterone

Duplicate plasma samples (50 μl) were extracted with 500 μl ethyl acetate (extraction efficiency 92%) and 400 μl aliquots of extract were assayed. Duplicate standards (0–800 pg/tube authentic testosterone in ethanol) or sample extracts were dried down in tubes containing approximately 5000 cpm, tritiated testosterone (Amersham) in ethanol. The antiserum was Sirosera C-6050 (Bioquest) used at a final dilution of 1:46,000 in phosgel (phosphate buffer, pH 7.6, with 1% gelatin). This antiserum cross-reacts with androsten-3β, 17β-diol (30%) and 5α-dihydrotestosterone (31%), other cross-reactivities being negligible. Two hundred microliters of antiserum was added to each tube (except non-specific binding tubes) and the assay was incubated at 4 °C overnight. Bound and free fractions were separated using dextran-coated charcoal (0.125%), and aliquots of the supernatants were counted in a Beckman LS 5801 liquid scintillation counter. Assay results were computed using logit-log modeling, and corrected for sample volume and extraction efficiency. The sensitivity of the assay was 2 pg testosterone (≈0.04 ng ml^{-1} plasma). Assay accuracy and precision were monitored by including in each assay replicates of three levels of commercially available human control serum (CON 6, DPC). Samples from 2001/2002 and 2003, respectively, were assayed in single assays: for both assays the testosterone concentrations measured in the three controls fell within the expected ranges and the intra-assay variation was <4%.

2.4. Radioimmunoassay of progesterone

The assay procedure was similar to that for testosterone except that plasma samples (50 μl) were extracted with 1 ml iso-octane (extraction efficiency 88–93%) and the entire extract was assayed. The antiserum was Sirosera C-9817 (Bioquest) used at a final dilution of 1:50,000 in phosgel. This antiserum cross-reacts with 11β-hydroxyprogesterone (22%), other cross-reactivities being negligible. The sensitivity of the assay was 2 pg progesterone (≈ 0.04 ng ml^{-1} plasma). Assay accuracy was again monitored using commercially available human control sera (CON 6, DPC), which fell within expected ranges. Samples from 2001/2002 and 2003, respectively, were assayed in single assays.

3. Results

Sixty-eight blood samples were obtained from 30 individual male echidnas over the period March 2000 to September 2003 (weight range 1.41–4.83 kg) and 38 samples were taken from 17 individual females over the period January 2002 to September 2003 (weight range 2.81–5.43 kg). Twenty-three animals (16 male, 7 female) are represented by only 1 sample but only 10 samples (7 male, 3 female) were taken from animals that were seen only once. Ten of the animals sampled (7 male, 3 female) had first been tagged in 1996, when we began work at the study site, and 3 animals had been observed in the field on more than 150 occasions. The maximum number of samples taken from an individual was 10 from a male, and 8 from a female.

Preliminary investigation of a range of samples from female echidnas confirmed that plasma oestradiol concentrations were less than 1 ng ml^{-1}, and virtually unmeasurable, as had been found previously in platypuses (Carrick et al., 1975) and echidnas (Dean, 2000). The highest values of both testosterone and progesterone were measured in samples taken in June, July, and August. Data are summarised in Figs. 1 and 3.

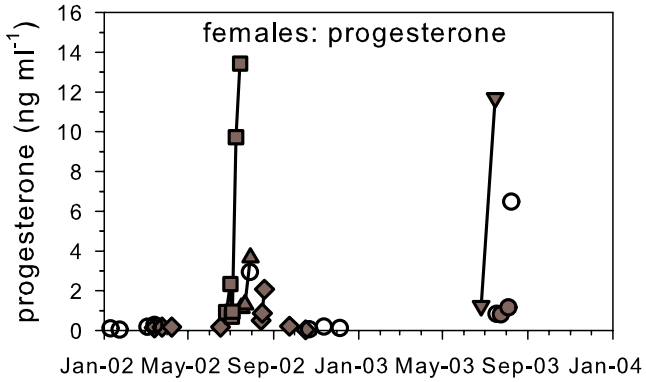

Fig. 1. Plasma progesterone concentrations of free-ranging female echidnas over two years. Shaded symbols indicate individual animals for which several samples were taken, lines join serial samples taken within a few days of each other.

3.1. Progesterone

For months other than June–August, the mean progesterone concentration was $0.18 \pm 0.12\,\mathrm{ng\,ml^{-1}}$ ($n = 14$). All blood samples taken from female echidnas in June–August had progesterone concentrations above $0.5\,\mathrm{ng\,ml^{-1}}$, except one from a female (5D5E) that was still hibernating when sampled on June 17. At this time she had a plasma progesterone of $0.18\,\mathrm{ng\,ml^{-1}}$ virtually identical to the concentration measured in April ($0.17\,\mathrm{ng\,ml^{-1}}$) before she entered hibernation. She subsequently mated and produced a young, and while all other female echidnas sampled during the period June–August engaged in reproductive activity, we do not know with certainty how many actually produced young. One sample was taken from female 5D5E during lactation approximately 6 weeks after egg-laying; by this time her plasma progesterone had fallen to $0.03\,\mathrm{ng\,ml^{-1}}$. The highest progesterone value measured was $13.4\,\mathrm{ng\,ml^{-1}}$ in a pregnant female (006F) for which we have a sequence of six measurements from 25 June to 15 July 2002 (Fig. 2). This female had been radio-tracked since February 1997, and had not had a young in this time. Her body temperature record from an implanted data logger shows she aroused from hibernation on June 25, reaching normal T_b (30 °C) at about 04:00. Ten hours later we found her dug into the soil with a male, and then on July 1 she was found in a different location dug in with the same male, as well as two others. On July 4, she was observed being followed by a fourth male, and on July 9 she was found in a hollow log with a fifth, but on July 11 and 12 she was alone. By July 15, when the last blood sample was taken she had a swollen pouch, and then on July 23 she had an egg in the pouch. Her body temperature record shows a decline in variability starting on July 17, suggesting egg-laying occurred on that date, or shortly after.

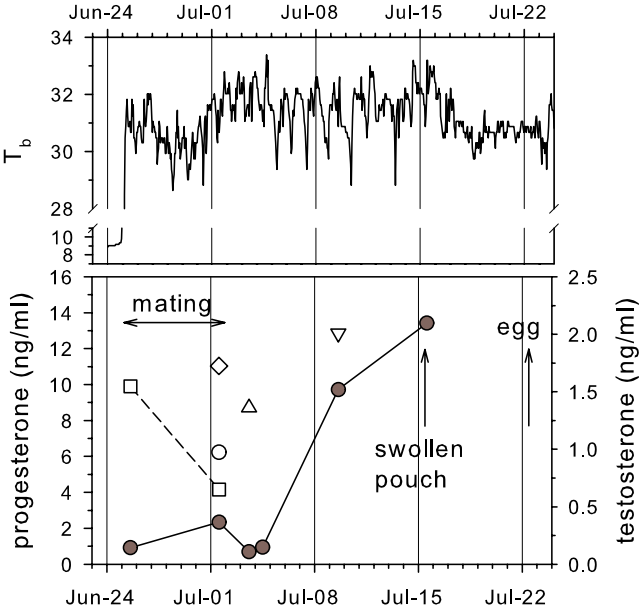

Fig. 2. Plasma progesterone concentrations of a female echidna (006F) from mating until shortly before egg-laying (shaded circles), and testosterone concentrations of 5 males found with her (open symbols). On July 22 she was found to have an egg in her pouch. The horizontal arrow indicates possible timing of mating estimated for a gestation period of 21–23 days. Top panel shows body temperature recorded with an implanted data logger. The decreased variability in T_b on July 17 suggests that egg-laying occurred on that day, in which case mating have occured within one day of arousal from hibernation.

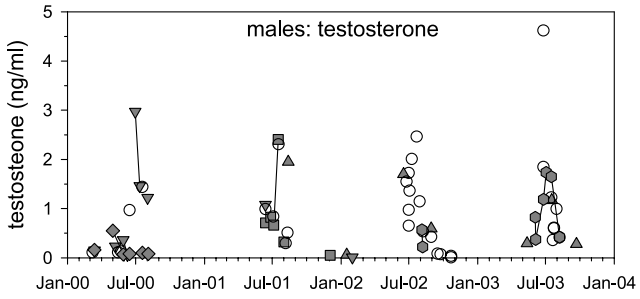

Fig. 3. Plasma testosterone concentrations of free-ranging male echidnas over four years. Shaded symbols indicate individual animals for which several samples were taken; lines join serial samples taken within a few days of each other.

3.2. Testosterone

For months other than June–August, the mean testosterone concentration was $0.09 \pm 0.05\,\mathrm{ng\,ml^{-1}}$ ($n = 13$). The highest plasma testosterone concentration measured was $4.62\,\mathrm{ng\,ml^{-1}}$. During June–August all active males except one had testosterone concentrations in excess of $0.2\,\mathrm{ng\,ml^{-1}}$ and were found in mating groups at some time during this period. The exception was a young male that had been classified as a juvenile 2 years previously, and which had a maximum testosterone of $0.1\,\mathrm{ng\,ml^{-1}}$. This echidna was not observed to be involved in any mating activity, although he was found in a mating

group the following year. Rismiller and McKelvey (2003) suggest that echidnas do not reach reproductive maturity until they are at least 5 years old, and this male could be classified as a sub-adult at the time of sampling.

Testosterone values from four hibernating males ranged from 0.07 to 0.55 ng ml^{-1}. There was no obvious relationship with time of year but the lowest values were from animals with the lowest body temperatures (Spearman rank correlation = 0.76, $P < 0.05$).

4. Discussion

These data demonstrate the strong seasonality of reproductive activity of echidnas and are consistent with plasma concentrations of echidna reproductive hormones measured by Dean (2000). Although her basal testosterone values (1 ng ml^{-1}) were higher than those obtained in the current study, peak values were similar. Basal progesterone values were similar, but Dean (2000) did not obtain any samples from animals known to be pregnant, and her peak values are much lower. Plasma concentrations of progesterone and testosterone in echidnas appear to be about half of those measured in platypus (Handasyde et al., 1992; Jabukowski et al., 1998; McFarlane and Carrick, 1992; New et al., 1998).

4.1. Testosterone and reproductive behaviour of male Tasmanian echidnas

Our data show the strong linkage between breeding behaviour and testosterone: all animals found in mating groups had significantly raised plasma testosterone concentrations. These values are generally lower than those measured by Dean (2000) in males in trains (3.7 ± 1.2 ng ml^{-1}, $n = 13$), but this could reflect a behavioural difference between sub-species. Tasmanian male echidnas do not spend as long in trains as Kangaroo Island males. However, one of the males for which we have detailed information (0000, hexagon symbols in Fig. 3) was observed in five different mating groups (or pairs) in 2003 over a period of 6 weeks, each at a different location within his home range, but the maximum testosterone concentration measured was only 1.7 ng ml^{-1}. Testosterone concentration may reflect the amount of prolonged inter-male competition: Dean (2000) found lower levels of plasma testosterone when fewer males were found in trains. While males with the highest testosterone may be the most successful in inter-male agonistic encounters (Dean, 2000) any suggestions about a relationship between testosterone concentrations and paternity need to be confirmed by genetic testing. Although Rismiller and McKelvey (2000) claim that females mate with the dominant male in a train, and mate only once, even in apparently monogamous pair bonded species of birds and mammals molecular techniques have demonstrated that surreptitious matings are much more common than was previously assumed (Fleischer, 1996).

5. Progesterone and pregnancy

Although all active female echidnas sampled in the period June–August had progesterone concentrations above the basal range (0.17 ± 0.11 ng ml^{-1}) the highest values were measured in females presumed to be pregnant, either because they were known to have later produced an egg, or because they had a swollen pouch when sampled. The highest value (13.4 nmol ml^{-1}) was the last of the series of measurements made on a pregnant female before she laid her egg (Fig. 2). As the gestation period is 21–23 days (Rismiller and McKelvey, 2000), and she aroused from hibernation on June 25, fertilization of this female must have occurred between June 25 and July 2. Jabukowski et al. (1998) have suggested that in platypus a pre-ovulatory progesterone surge could play an essential role in development of the ovarian follicle and trigger the pre-ovulatory LH surge, and it is possible that the rise in progesterone observed on July 1 in Fig. 2 was pre-ovulatory, as also occurs in some viviparous reptiles (Jones and Baxter, 1991). However, if egg-laying occurred on July 17 as the T_b record suggests, and if the gestation period is 21–23 days, fertilisation would have occurred before this. The major rise in progesterone is presumably associated with the development of the corpus luteum. Although Hill and Gatenby (1926) claimed that the regression of the monotreme corpus luteum begins well before the egg is laid, this seems unlikely as our last blood sample was probably taken only 1–2 days before egg-laying, and Hughes and Carrick (1978) found normal corpora lutea in a full-term platypus. We do not have any data from females with an egg in the pouch, and so we do not know how rapidly plasma progesterone declines after egg-laying. We obtained one sample from a lactating female 37 days after the estimated date of egg-laying, and this animal had a progesterone concentration of 0.03 ng ml^{-1}, within the basal range.

5.1. Hibernation and the timing of reproductive activity

Testosterone concentrations are at their highest in June–July and progesterone highest in July–August. From our data and the data of Dean (2000), the highest levels of testosterone appear to occur when male echidnas are in mating groups. Testosterone concentrations are basal during late hibernation, and one likely reason why males arouse from hibernation before females (Nicol and Andersen, 2002) is that several weeks of euthermia are required for testicular growth and spermatogenesis, as is the case for ground

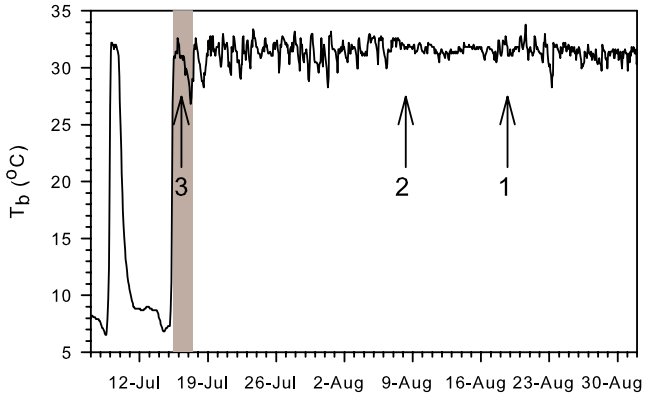

Fig. 4. Body temperature of a female echidna (5D5E) recorded with an implanted data logger. The recording shows that her final arousal from hibernation was mid-morning on July 15, 1999. On July 17 (indicated by arrow 3) she was in a hollow tree stump with another echidna. On July 27 she had developed a pouch, and on August 18 (indicated by arrow 1) she had both egg shell and a young in the pouch, indicating the young had been very recently hatched. Assuming 10.5 days for incubation of the egg, it would have been laid at the time indicated by arrow 2, and assuming a 21–23 day gestation period, mating must have occurred in the shaded period, i.e., within no more than 2.5 days of the arousal from hibernation. Note the reduction in variability of T_b between arrows 1 and 2, when the mother was in the nursery burrow incubating the egg. Redrawn from Nicol and Andersen (2002).

squirrels (Barnes, 1996). How much testicular growth occurs before entry into hibernation is not clear; Griffiths (1978) reports that adult echidnas collected in southeast Australia had the lowest testes weights from October to March, but large testes and spermiogenesis were observed in some animals collected in April and May, and in most animals collected in July, August, and September. However, at that time very little information was available on the occurrence of hibernation in free-living echidnas, and it not known whether those males showing spermiogenesis in April and May had hibernated. The minimum time we have observed between arousal from hibernation and male reproductive activity is about 3 weeks, as judged by a recording of activity from a logger attached to an animal (Nicol et al., 2004). By contrast, females may mate within days of the final arousal from hibernation Figs. 2 and 4, and inter-male competition would be expected to strongly select for early male arousal from hibernation.

6. Conclusions

We now have data on the seasonal variations in progesterone (Fig. 1) and testosterone (Fig. 3) in an echidna population, and have established a clear correlation between high testosterone and the period of male mating behaviour. We have also shown high progesterone to be correlated with pregnancy. High progesterone levels were also noted to be correlated with a swollen pouch, and probably with the construction of a nursery burrow. The variation between individuals in the times at which they enter and arouse from hibernation, and the very significant variation an individual echidna may show on consecutive years (Nicol and Andersen, 2002), means that a clear understanding of the relationship between hibernation and reproduction, and the hormonal control of reproduction will require more longitudinal studies on individual animals. Because echidnas do not hibernate normally in captivity (Nicol and Andersen, 1993), and have only on very few occasions been known to reproduce in captivity (Rismiller, 1999) this work must be carried out on echidnas in the wild. However, echidnas are small, (adult weight range 2–5 kg), cryptic, semi-fossorial, and largely nocturnal (Nicol et al., 2004). They are also a fully protected species. Individuals can only be sampled sequentially by radio tracking, and even when located, they are often in inaccessible locations. This means that considerable time and effort will be required to obtain a more complete understanding of the time course of the hormonal control of reproductive activity of echidnas, and to establish whether there really is a pre-ovulatory rise in progesterone, how plasma progesterone changes immediately before and after birth, and the hormonal correlates of spermatogenesis.

Acknowledgments

We thank Megan Richards, Christina Vedel-Smith, and Anna Reye for their invaluable assistance in the field. Adrian Bradley performed the testosterone analyses on the plasma samples from 2000, and Heather Hesterman assisted with the other analyses. Randy Rose contributed to the planning of the 2000 study. We are grateful to the McShane family of 'Lovely Banks' for allowing us to carry out this work on their property. Financial support was provided by the Australian Research Council, the University of Tasmania Internal Research Grant Scheme, and the National Geographic Committee for Research and Exploration. This work was carried out under permit from the Tasmanian Department of Primary Industries, Water and Environment, and the University of Tasmania Animal Ethics Committee, and complies with the Tasmanian Animal Welfare Act (1993) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2005.05.013.

References

Barnes, B.M., 1996. Relationships between hibernation and reproduction in male ground squirrels. In: Geiser, F., Hulbert, A.J., Nicol, S.C. (Eds.), Adaptations to the Cold: Tenth International Hibernation Symposium, vol. University of New England Press, Armidale, pp. 71–80.

Beard, L.A., Grigg, G.C., 2000. Reproduction in the short-beaked echidna, *Tachyglossus aculeatus*: field observations at an elevated site in south-east Queensland. Proc. Linnean Soc. New South Wales 122, 89–99.

Beard, L.A., Grigg, G.C., Augee, M.L., 1992. Reproduction by echidnas in a cold climate. In: Augee, M.L. (Ed.), Platypus and Echidnas, vol. Royal Zoological Society NSW, Mosman, pp. 93–100.

Carrick, F.N., Drinan, J.G., Cox, R.I., 1975. Progestagens and oestrogens in peripheral plasma of the platypus. J. Reprod. Fertil. 43, 375–376.

Dean, K.M., 2000. Aspects of the reproductive biology of the echidna, *Tachyglossus aculeatus*. PhD thesis, Department of Zoology, University of Melbourne.

Fleischer, R., 1996. Application of molecular methods to the assessment of genetic mating systems in vertebrates. In: Ferraris, J., Palumbi, S. (Eds.), Molecular Zoology, vol. Wiley-Liss, New York.

Gemmell, R.T., 1995. A comparative study of the corpus luteum. Reprod. Fertil. Dev. 7, 303–312.

Griffiths, M., 1978. The Biology of Monotremes. Academic Press, New York.

Griffiths, M., 1989. Tachyglossidae. In: Walton, D.W., Richardson, B.J. (Eds.), Fauna of Australia, Mammalia, vol. 1B. Australian Government Publishing Service, Canberra, pp. 407–435.

Grigg, G.C., Beard, L.A., Augee, M.L., 1989. Hibernation in a monotreme, the echidna *Tachyglossus aculeatus*. Comp. Biochem. Physiol. A 92, 609–612.

Handasyde, K.A., McDonald, I.R., Evans, B.K., 1992. Seasonal changes in plasma concentration of progesterone in free-ranging platypus. In: Augee, M.L. (Ed.), Platypus and Echidnas, vol. Royal Zoological Society of New South Wales, Sydney, pp. 75–79.

Higgins, D.P., Tobias, G., Stone, G.M., 2004. Excretion profiles of some reproductive steroids in the faeces of captive female short-beaked echidna (*Tachyglossus aculeatus*) and the long-beaked echidna (*Zaglossus* sp.). Proc. Linn. Soc. New South Wales 125, 279–286.

Hill, C.J., 1941. The development of the Monotremata. V. Further observations on the histology and secretory activities of the oviduct prior to and during gestation. Trans. Zool. Soc. Lond. 25, 1–31.

Hill, J.P., 1933. The development of the Monotremata. II. The structure of the egg-shell. Trans. Zool. Soc. Lond. 26, 443–476.

Hill, J.P., Gatenby, J.B., 1926. The corpus luteum of the Monotremata. Proc. Zool. Soc. Lond. 47, 715–763.

Hughes, R.L., Carrick, F.N., 1978. Reproduction in female monotremes. Aust. Zool. 20, 233–253.

Jabukowski, J.M., New, N.P., Stone, G.M., Jones, R.C., 1998. Reproductive seasonality in female platypuses, *Ornithorhynchus anatinus*, in the Upper Barnard River, New South Wales. Aust. Mammal. 20, 207–213.

Jones, R.E., Baxter, D.C., 1991. Gestation, with emphasis on corpus luteum biology, placentation, and parturition. In: Pang, P.K.J., Pang, P.K.T., Schreibman, M.P. (Eds.), Vertebrate Endocrinology: Fundamental and Biomedical Implications, Part A. Reproduction, vol. 4. Academic Press, San Diego, pp. 205–302.

McFarlane, J.R., Carrick, F.N., 1992. Androgen concentrations in male platypuses. In: Augee, M.L. (Ed.), Platypuses and Echidnas, vol. Royal Zoological Society of New South Wales, Sydney, pp. 69–74.

Millis, A.L., Bradley, A.J., 2001. Reproduction in the squirrel glider, *Petaurus norfolcensis* (Petauridae) in south-east Queensland. Aust. J. Zool. 49, 139–154.

New, N.P., Jabukowski, J.M., Stone, G.M., Jones, R.C., 1998. Seasonal pattern of androgen secretion in the male platypus, *Ornithorhynchus anatinus*, in the Upper Barnard River, New South Wales. Aust. Mammal. 20, 8–9.

Nicol, S., Andersen, N.A., 2002. The timing of hibernation in Tasmanian echidnas: why do they do it when they do?. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 131, 603–611.

Nicol, S.C., Andersen, N.A., 1993. The physiology of hibernation in an egg-laying mammal, the echidna. In: Carey, C., Florant, G.F., Wunder, B.A., Horwitz, B. (Eds.), Life in the Cold III: Ecological, Physiological, and Molecular Mechanisms, vol. Westview Press, Boulder, CO, pp. 55–64.

Nicol, S.C., Andersen, N.A., 1996. Hibernation in the echidna: not an adaptation to cold?. In: Geiser, F., Hulbert, A.J., Nicol, S.C. (Eds.), Adaptations to the Cold: Tenth International Hibernation Symposium, vol. University of New England Press, Armidale, pp. 7–12.

Nicol, S.C., Vedel-Smith, C., Andersen, N.A., 2004. Behavior, body temperature and hibernation in Tasmanian echidnas (*Tachyglossus aculeatus*). In: Barnes, B.M., Carey, H.V. (Eds.), Life in the Cold 2004, vol. 27. Biological Papers of the University of Alaska, Fairbanks, Alaska, pp. 149–157.

Oates, J.E., Bradshaw, F.J., Bradshaw, S.D., Lonsdale, L.A., 2002. Sex identification and evidence of gonadal activity in the short-beaked echidna (*Tachyglossus aculeatus*) (Monotremata: Tachyglossidae): non-invasive analysis of faecal sex steroids. Aust. J. Zool. 50, 395–406.

Oftedal, O.T., 2002. The origin of lactation as a water source for parchment-shelled eggs. J. Mammary Gland Biol. Neoplasia 7, 253–266.

Renfree, M.B., Shaw, G., 1999. Marsupials. In: Nobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction, vol. 3. Academic Press, San Diego, pp. 104–114.

Rismiller, P.D., 1999. The echidna, Australia's enigma. Hugh Lauter Levin Associates, Connecticut.

Rismiller, P.D., McKelvey, M.W., 1996. Sex, torpor and activity in temperate climate echidnas. In: Geiser, F., Hulbert, A.J., Nicol, S.C. (Eds.), Adaptations to the Cold: Tenth International Hibernation Symposium, vol. University of New England Press, Armidale, pp. 23–30.

Rismiller, P.D., McKelvey, M.W., 2000. Frequency of breeding and recruitment in the short-beaked echidna, *Tachyglossus aculeatus*. J. Mammal. 81, 1–17.

Rismiller, P.D., McKelvey, M.W., 2003. Body mass, age and sexual maturity in short-beaked echidnas, *Tachyglossus aculeatus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 136, 851–865.

Swain, R., Jones, S.M., 2000. Facultative placentotrophy: half-way house or strategic solution?. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 127, 441–451.

Temple-Smith, P., Grant, T., 2001. Uncertain breeding: a short history of reproduction in monotremes. Reprod. Fertil. Dev. 13, 487–497.