

Immunoglobulin genes of the platypus and echidna

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Prior studies on the immune system of the monotremes have been limited to the characterisation of lymphoid tissues and of antibody responses. Over the past few years, we have begun the characterisation of the monotreme immune system at the molecular level. In this paper, we will review all prior studies on the monotreme immune system, as well as summarising our own findings. In particular, we will focus on the immunoglobulin repertoire of the monotremes.

Immunoglobulin molecules consist of two identical heavy chains and two identical light chains. Both the heavy and the light chains are composed of a constant region and a variable region. The number and identity of immunoglobulin genes varies greatly between mammals and non-mammals. The characterisation of IgM, IgG, IgA and IgE from the platypus and echidna shows that the monotreme immunoglobulins are structurally identical to the immunoglobulin isotypes found in marsupials and eutherians, but not birds and reptiles. Monotreme IgG and IgA constant regions contain three domains plus a hinge region and monotremes utilise IgE rather than IgY. Analysis of monotreme heavy chain variable region diversity suggests that the platypus uses a single VH clan, while the short-beaked echidna utilises 4 distinct VH families which segregate into all three mammalian VH clans. Both lambda and kappa light chains have been isolated from both the platypus and echidna.

Phylogenetic analysis of the immunoglobulin gene sequences provides strong support for the traditional Theria hypothesis, which suggests that monotremes diverged prior to the separation of the marsupials and eutherians. IgM has proven to be a useful marker for estimating the time of divergence of mammalian lineages. We estimate that the monotremes and therians last shared a common ancestor ~170 million years ago, marsupials and eutherians ~130 million years ago, Australian and American marsupials ~65 million years ago and the platypus and echidna about 22 million years ago.

Keywords: Monotreme, Platypus, Echidna, Immunoglobulin, Constant region, Variable region, Evolution

**Aerobic dive limit and the organisation of the dive cycle in the platypus
*Ornithorhynchus anatinus***

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We investigated the aerobic dive limit and the organisation of the dive cycle of platypuses (*Ornithorhynchus anatinus*) living at a sup-alpine Tasmanian lake. Individual platypuses were equipped with combined data-logger-transmitter packages measuring dive depth. Mean dive duration was 31.3 seconds with 72% of all dives lasting between 18 and 40 seconds. Mean surface duration was 10.1 seconds. Mean dive depth was 1.28 m with a maximum of 8.77 m. Up to 1600 dives per foraging trip with a mean of 75 dives per hour were performed. Aerobic dive limit was estimated by consideration of post-dive surface intervals versus dive durations. Only 15% of all dives were found to exceed the estimated aerobic dive limit of 40 seconds indicating mainly aerobic diving in the species. While foraging, platypuses followed a model of optimised recovery time - the optimal breathing theory. Total bottom duration per day is proposed as a useful indicator of foraging efficiency and hence habitat quality in the species.

Is the platypus X chromosome inactivated?

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Although the sex chromosomes of monotremes are complex and incompletely known, it is clear that a large X chromosome is present in one copy in males and two in females. This means that some thousands of genes are present in one dose in males and two in females. We would therefore expect dosage compensation to be essential in platypus.

Dosage compensation for X-borne genes is achieved in eutherians and marsupials via inactivation of one X chromosome in female somatic cells. In eutherians, X inactivation is random, stable and complete. It appears to be under the control of an X inactivation centre known as *XIST*. Marsupials diverged from eutherians 130-150 million years ago and display a more simplistic form of X inactivation. In these mammals, X inactivation is characterized by being paternal, tissue specific and incomplete. Since X inactivation occurs in both of these distantly related mammalian groups, a basic mechanism is likely to have been in place in a common ancestor prior to their divergence. Monotremes, having diverged from eutherians 170 million years ago, may assist in unravelling the evolution of X chromosome inactivation mechanisms. Of particular interest is the evolution of the *XIST* gene. Genes flanking *XIST* in eutherians (*MNK*, *CDX4*, *XPCT*) have been cloned in the platypus to determine the chromosomal location of this region in monotremes. Additionally, it is anticipated that the cloning of these genes will now assist in the discovery of the poorly conserved *XIST* gene (or its progenitor) in the platypus by chromosome walking. Interestingly, *CDX4* and *XPCT* have been mapped to an autosomal location on platypus chromosome 6. Three genes representative of different regions of the platypus X chromosome, one from the pseudoautosomal region of Xp (*UBE1*) and two from Xq (*AR*, *G6PD*) have also been isolated. Semi-quantitative RT-PCR carried out on these genes indicates that there is dosage compensation in female platypuses but it remains to be determined if this is achieved by the inactivation of an X chromosome.

Ventilation in the monotremes: effects of lifestyle and the evolution of the mammalian breathing pattern

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The rates of oxygen consumption, ventilation and breathing patterns in the platypus (*Ornithorhynchus anatinus*) and echidna (*Tachyglossus aculeatus*) together with their responses to hypoxia and hypercapnia are examined with respect to their lifestyles. Both species possess a reduced ventilatory response to hypoxia and hypercapnia as previously demonstrated for diving or burrowing species, though a general reduction in sensitivity to carbon dioxide has previously been reported for marsupials irrespective of lifestyle. The platypus shows modification of its breathing pattern with a high inspiratory airflow rate, which is typical of diving species. Comparison to marsupials and eutherians reveals that the air convection requirement (ventilation/metabolic rate) is an interspecies 'constant' across the three mammalian infraclasses, despite reported differences in body temperature and rates of oxygen consumption. In order for the changes in ventilation to parallel the increases in metabolic rate that occurred during the evolution of mammalian homeothermy, it would appear that the respiratory cycle was shortened largely through the removal of an end inspiratory pause and with an increase in respiratory 'drive'.

Monotreme genes and genomes

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Monotremes are really the last frontier of research on mammalian genomes. Their phylogenetic position means that they can serve as mammalian outgroup to answer questions about the evolution of genome organization, function and evolution.

For many years now, our group has studied platypus and echidna chromosomes and gene arrangements, concentrating on the sex chromosomes. Platypus and echidna have different chromosome numbers, but share an extraordinary translocation chain (unique in mammals) made up of unpaired chromosomes. This chain includes an obvious X chromosome, which is the same in platypus and echidna. We have performed comparative mapping on a number of genes which are located on the human X chromosome. We found that one set of genes is on the X in all mammals, and therefore represents an ancient mammalian X, and another set is X-borne only in eutherians, representing a region added recently in the eutherian lineage. Monotremes have been a critical outgroup in these studies.

The monotreme X chromosome is present in two copies in females and a single copy in males, so there might be expected to be a mechanism to compensate for dosage differences in males and females. We are studying the amount of transcript from X-borne and autosomal genes in order to determine whether X chromosome inactivation occurs.

Sex determination in monotremes is also utterly unknown. There is no clear evidence of a Y chromosome with male-specific regions, and we have been unable to detect a platypus homologue of the *SRY* gene that is testis determining in eutherians and marsupials.

To help answer these questions, and to provide data of use for phylogenetic footprinting, we have collaborated on constructing a BAC library, and there is serious talk about sequencing the platypus genome in the near future.

Body temperature of the long-beaked echidna (*Zaglossus bruijini*) in captivity

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The routine occurrence of both short term (daily) and long term torpor (hibernation) in short-beaked echidnas, but not platypus, raises questions about the third monotreme, the New Guinea long-beaked echidna, *Zaglossus bruijini*. Accordingly, body temperatures (T_b) were measured with implanted data loggers over three and a half years in two captive *Z. bruijini* at Taronga Zoo, Sydney. T_b cycled daily over 2-4°C, with no departures from this pattern to suggest either daily torpor or hibernation. Frequency histograms of T_b were symmetrical. In contrast, two short-beaked echidnas, *Tachyglossus aculeatus*, monitored during the same period in similar pens, showed extended periods of low T_b in the cooler months (hibernation) and frequency histograms of T_b (excluding hibernation) skewed towards lower temperatures, reflecting the known occurrence of short periods of torpor at any time of the year in *T. aculeatus*. The modal T_b of both longbeaks was 31°C, similar to the captive short beaks (32°C) and wild echidnas in their 'non-hibernation' periods (30-32°C), and to platypus (32°C). This suggests that a T_b of 30-32°C is characteristic of normothermic monotremes. Whether torpor or hibernation occurs in *Zaglossus* in the wild is unknown. However, because the environment in this study was conducive to hibernation in shortbeaks, which do not easily enter torpor in captivity, its occurrence in wild *Zaglossus* is unlikely.

Chromosome painting in platypus

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Monotremes (platypus and echidna) are unique mammals, distantly related to human and featuring a weird mix of characters that link them to birds and reptiles. The chromosomes of monotremes are equally unique and weird. Several chromosomes (7 in platypus and 9 in echidna, including the sex chromosomes) have no partners, and form a chain in male meiosis that suggests serial translocation. In humans a single translocation causes sterility and chromosome abnormalities in the offspring, so how monotremes are fertile while maintaining heterozygosity of several translocated chromosomes is completely mysterious. Equally mysterious is how monotremes determine sex. A Y chromosome has yet to be identified and there appears to be no sex determining SRY gene.

We are using molecular cytogenetics techniques, mainly chromosome painting, to discover how the monotreme karyotype is organized in mitosis, meiosis and sperm and to identify the sex chromosome system. An important step towards the identification of the sex chromosomes and the translocated chromosomes is the generation of so called chromosome paints. These DNA libraries contain the DNA of single isolated whole chromosomes which could be used as a hybridisation probe. Chromosome painting is a widely used technique to unravel chromosomal homology between species that diverged not more than 20-40 Million years. Chromosome paints are also used in human pathology to identify chromosomal abnormalities like translocations. In the platypus we are using chromosome paints to identify chromosomal homologies of the unpaired chromosomes including the sex chromosomes. Although the observation of a meiotic translocation chain implies translocation heterozygosity, the translocations have not been identified yet. A set of whole chromosome paints has been generated by flow sorting platypus chromosomes and these chromosome-specific paints are hybridized onto metaphase chromosomes of male and female platypus as well as echidna. For the first time it is now possible to identify the translocations in the platypus and to compare them in male and female animals. Moreover, chromosome painting allows to follow up these chromosomes in meiosis, sperm formation and mature sperm. The comparison between platypus and echidna will show if the same chromosomes are involved in these translocations, and explain the different number of the unpaired elements in the two species.

Our set of chromosome-specific paints contains larger platypus chromosomes 1, 4, 6 and X as well as twelve smaller chromosomes. The larger chromosomes show no differences between male and female chromosomes. The X chromosome paint, however, stains one X and a small chromosome in males, and two identical XX chromosomes in female. This would be consistent with an XX/XY sex chromosomes system normal for most mammals, except that the "Y" is completely homologous to

the short arm of the X and seems therefore to contain no male-specific region. Other chromosome paints hybridise to more than one chromosome pair which are strong candidates for unpaired elements. The localization of these paints onto the meiotic chain will test this, and provide information about the position of the translocation in the chain.

The advances in molecular cytogenetics that have been achieved during the last decade, especially chromosome painting, will therefore allow us to resolve the weird monotreme karyotype, to determine how the sex chromosomes and the translocated chromosomes are organized in mitosis, and segregated into meiosis and sperm to produce viable offspring.

Seasonal patterns in glucocorticoids in relation to reproduction in free-ranging platypus (*Ornithorhynchus anatinus*)

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The platypus is a seasonal breeder, with mating and egg laying occurring during late winter and early spring. At this time energy expenditure is likely to increase because animals are spending some of their time engaged in reproductive activities such as searching for mates or (in females) construction of breeding burrows. In the southern parts of its distribution reproduction in the platypus coincides with strongly seasonal environmental patterns, including cold water temperatures and river flooding. These factors lead to the prediction that animals would need to mobilise additional energy to meet the increased demands of thermoregulation and breeding season activity during this period. Glucocorticoids from the adrenal gland play a major role in energy mobilisation. We collected blood samples from adult female (n=44) and male (n=32) free-ranging platypus in Victoria at approximately monthly intervals throughout the year and measured glucocorticoid concentrations via radio-ligand assay. We observed a strong seasonal pattern in plasma glucocorticoids. In males, mean plasma glucocorticoid concentrations rose from 129 ± 29 nmol/L in the non-breeding season, to 270 ± 35 nmol/L in the breeding season. Females showed a similar pattern with a mean of 104 ± 16 nmol/L in the non-breeding season and 236 ± 34 nmol/L in the breeding season. These data, and the decline in tail fat stores that occur towards the end of the mating period, are consistent with the idea that platypus experience high energy demands during this phase of reproduction. One interesting result from this study was that plasma glucocorticoid concentrations of females sampled during the period when most adult females are lactating, November to January, (a time when one might predict energy demands on females to be high) were relatively low and similar to those in females sampled in the non-breeding, non-lactation period (March to June): 104 ± 24 nmol/L and 114 ± 20 nmol/L respectively. These data suggest that the combination of thermal stress (imposed by cold winter conditions) and breeding season activity are more significant stressors for platypus than factors such as lactation in females.

The Anatomy of the Cerebral Cortex of the Echidna (*Tachyglossus aculeatus*)

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We have used Nissl staining, immunocytochemistry for neurofilament protein, Golgi staining and electron microscopy to study the structural organization of the cerebral cortex of the echidna. All 6 echidnas used in this study were obtained from the wild under licence and were anaesthetized by intramuscular injection of pentobarbitone (35 mg/kg) before perfusion with the appropriate aldehyde fixatives. The cerebral cortex of the echidna is notable for its extensive folding and the positioning of major functional areas towards its caudal extremity. The gyrification of the echidna cortex is comparable to prosimians and we found that cortical thickness and neuronal density are similar to that seen in rodents and carnivores. On the other hand, Golgi-Colonnier impregnations showed that many pyramidal neurons in the cerebral cortex of the echidna are atypical with inverted somata and short or branching apical dendrites. Nevertheless, all other broad classes of neurons found in eutherian cortex also appear to be present in the echidna, suggesting that the major classes of cortical neurons evolved prior to the divergence of proto- and eutherian lineages. Dendritic spine density on dendrites of echidna pyramidal neurons in somatosensory cortex and apical dendrites of motor cortex pyramidal neurons and the diameter of apical dendrites were found to be lower than in eutheria. On the other hand, synaptic morphology, density and distribution in somatosensory cortex were found to be similar to that in eutheria. In summary, although the echidna cerebral cortex displays some structural features, which may limit its functional capacities (e.g. lower spine density on pyramidal neurons), in most structural parameters (e.g. gyrification, cortical area and thickness, neuronal density and types, synaptic morphology and density), it is comparable to eutheria.

Development of a predictive equation for field metabolic rate in the echidna: correlations between VO₂, heart rate, Tb and activity

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Using open-flow respirometry, this paper examines the inter-relationships between metabolic rate and activity, body temperature (T_b) and heart rate (HR) in eight echidnas (*Tachyglossus aculeatus*) at two ambient temperatures ($T_a = 12$ and 25°C) and develops predictive equations for use in estimating field metabolic rates. Predictive equations were developed for use in studies of torpor and hibernation, thus the variables chosen reflect those measurable in the field. The mean VO_2 for each echidna at rest (no visible movement) was significantly lower than when active (visible movement), and the mean VO_2 recorded at 12°C (below the reported thermoneutral zone of $20 - 30^\circ\text{C}$) for each animal was higher than at $T_a 25^\circ\text{C}$. Mean resting VO_2 in each animal at $T_a 12^\circ\text{C}$ was equal to or slightly lower than at $T_a 25^\circ\text{C}$. T_b did not remain constant during experiments and ranges were slightly lower at $T_a 12^\circ\text{C}$ ($23 - 28^\circ\text{C}$) than at 25°C ($28 - 33^\circ\text{C}$). There was no correlation between resting VO_2 and whether an animal was warming or cooling and the relationship between VO_2 and T_b was not consistent between individuals. We saw no sign that echidnas increase resting metabolism to defend a set T_b at low T_a , suggesting that they do not conform well to the traditional model of thermoregulation in endotherms. The data used to develop predictive equations included literature values for torpid echidnas because they did not enter torpor in the present study. Several multivariate approaches were used. The best-fit models for each individual were achieved when data from echidnas at rest and active were treated separately (mean $r = 0.70$). Heart rate explained more than 50% of the variation in VO_2 in all but one of the best-fit models. We concluded that monitoring heart rate provides a useful method for estimating VO_2 in echidnas, which can be improved significantly by the inclusion of activity and/or T_b .

**Sizes of testes and venom glands in male Tasmanian platypuses,
Ornithorhynchus anatinus, as influenced by season of the year, maturity
and bodyweight**

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Testes and venom glands dissected from accidentally-killed platypuses were weighed and /or measured. The results showed a correlation between testis size/weight and venom gland size/weight. Testis size was affected by season and maturity of the platypuses. The data suggest that the breeding season for Tasmanian platypuses is later than that on mainland Australia.

Control of breathing during hibernation in the echidna

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Resting non-hibernating echidnas are characterised by low metabolic rates, but also have a very low respiratory frequency leading to a low and quite variable respiratory minute volume, often resulting in low levels of arterial oxygen and high CO₂. As the echidna seems to lie at one physiological extreme among the hibernators, in terms of its large size and low metabolism and ventilatory requirement when not hibernating, a study of control of breathing during hibernation in echidnas could provide a useful test of the generality of various models.

We studied breathing patterns and the control of respiration in 6 echidnas. Echidnas will not hibernate when disturbed and so breathing pattern was measured non-invasively from chamber pressure. Hibernating echidnas showed episodic breathing with long apneas broken by bursts of breathing. A typical burst contained about 30 breaths. Between these main bursts were much smaller bursts of 1-4 breaths. Increasing CO₂ levels in the inspired air increased the number of breaths in a burst, eventually leading to continuous breathing. Interburst breaths appear to be controlled by the peripheral chemoreceptors: hypoxia increased interburst breaths, and decreased burst length, while hyperoxia increased the apneic period, but increased burst length.

Using respiratory frequency as an index of total ventilation, oxygen had little effect on total ventilation in hibernating animals, while carbon dioxide was a strong stimulant. Oxygen levels did however have a strong effect on the breathing pattern.

Our results suggest that the onset of respiration is strongly dependent on the O₂ level, while the continuation of a burst is strongly dependent on CO₂. The individual breaths that occur between bursts are initiated by low PO₂. Whether a breath is a single breath or a burst seems to depend on the CO₂ level, and the difference between breathing and apnoeic thresholds, which appears to be strongly influenced by O₂.

Electrolocation in the platypus?

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Our work has shown that some cells in the platypus cerebral cortex respond to both electrosensory and mechanical inputs from the bill. Jack Pettigrew has suggested that these neurones may be tuned to respond preferentially to electroreceptor and mechanoreceptor inputs arriving at preferred intervals. The electrical signal generated by a moving prey item such as a shrimp would reach the platypus first. Then, at a delay depending on how far away the prey was, the mechanical disturbance would arrive. The appropriately tuned cells would allow computation of the prey's distance. Other information about motion of the prey and its direction would be provided by the stimulus gradients across the array of sensory receptors in the bill. Such a mutual co-operation between two different sensory systems is unique. This could reflect the evolution of the platypus' electrosensory system, which probably has a projection pathway to the brain identical to that for transmission of tactile information. It could therefore be argued that the existence of a separate electric sense in the platypus has not been demonstrated unequivocally, and perhaps the platypus perceives the electrosensory input as a tactile sensation.

Keywords: Afferent, Cerebral cortex, Electroreceptor, Mechanoreceptor, Platypus, Prey, Somatosensory, Tactile

**Growth, body mass and sexual maturity in short-beaked echidnas,
*Tachyglossus aculeatus***

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During the course of this 12 year study body masses of 11 hatchling echidnas < 48 hrs old and growth rates in 25 pouch young between the ages of 5 and 60 days were recorded. Pouch young increased body mass 3500% in the first 30 days. Mass doubled again before young were placed in a burrow at 50 to 60 days of age. There was a positive correlation between the body mass of the female and that of her young at weaning. Twenty-four subadult echidnas were located, tagged and radio tracked. From these, the body masses of 10 were measured up to the age of sexual maturity, ie when first encountered in a courtship train. One female was courted and mated at 5 years of age. All other animals were found in courtship at 6 to 12 years of age. After positive determination of gender, no difference between initial weights of male and female subadults was found. However mean body mass at sexual maturity was significantly greater in females. There was a positive correlation between age of sexual maturity and body mass in females.

Keywords: Monotreme, Echidna, Growth, Age, Sexual maturity

Tactile neural mechanisms in the echidna

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Monotremes, perhaps more than any other order of mammals, display an enormous behavioural reliance upon the tactile senses. In the platypus this is manifest most strikingly in the special importance of the bill as a peripheral sensory organ, an importance confirmed by electrophysiological mapping that reveals a vast area of the cerebral cortex allocated to the processing of tactile inputs from the bill. Although behavioural evidence in the echidna suggests a similar prominence for tactile inputs from the snout there is also a great reliance upon the distal limbs for digging and burrowing activity, which points to the importance for the echidna of tactile information from these regions.

In the present study we have investigated the peripheral tactile neural mechanisms in the forepaw of the echidna in order to establish the extent of correspondence or divergence that has emerged over the widely different evolutionary paths taken by monotreme and placental mammals. Electrophysiological recordings were made from single tactile sensory nerve fibers isolated in fine strands of the median or ulnar nerves of the forearm. Controlled tactile stimuli applied to the forepaw glabrous skin permitted an initial classification of tactile sensory fibres into two broad divisions, according to their responses to static skin displacement. One displayed *slowly adapting (SA)* response properties, while the other showed a selective sensitivity to the dynamic components of the skin displacement. The broad *SA* class included fibres with low-thresholds and punctate receptive fields and other fibres that had either larger cutaneous fields or an association with the base of the claws. The purely dynamically-sensitive tactile fibers could be subdivided according to vibrotactile sensitivity and receptive field characteristics into a *rapidly adapting (RA)* class, sensitive to low frequency (≤ 50 Hz) vibration, that resembled a corresponding *RA* class in placental species, and another class, sensitive to a broader range of vibrotactile frequencies (~ 50 -300 Hz), that may represent a monotreme equivalent of the *Pacinian corpuscle (PC)-related* fiber class of placental mammals. The differential tactile sensitivity of the three principal fiber classes and their individual coding characteristics, determined by quantitative stimulus-response analysis, indicate first, that this triad of fiber classes can subservise high-acuity tactile signalling from the echidna footpad, and second, that peripheral tactile sensory mechanisms are highly conserved across evolutionarily-divergent mammalian orders.

Keywords: Monotreme, Somatosensory system, Echidna, Tactile receptors, *Tachyglossus aculeatus*, Evolution and sensory nerve function, Tactile sensory function

**Field energetics of free-living, lactating and non-lactating echidnas
(*Tachyglossus aculeatus*)**

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We measured daily energy expenditure (DEE) and water flux rates of 7 free-living echidna females - 3 lactating and 4 non-lactating - using doubly labelled water ($D_2^{18}O$). Two females (one lactating, one non-lactating) had temperature-sensitive data loggers implanted to record body temperature (T_b). The non-lactating echidnas were on average significantly heavier ($3,353 \pm 236$ g) than the lactating females ($2,700 \pm 8$ g) during the equivalent time period. The mean water flux rate of lactating echidnas (156.5 ± 22.4 ml day⁻¹) did not differ significantly from that of non-lactating females (173.8 ± 20.3 ml day⁻¹). The mean DEE of echidnas that were lactating was 692.2 ± 81.1 kJ day⁻¹, which was not significantly different from the mean DEE of non-reproductive control females (753.2 ± 10.9 kJ day⁻¹). Average T_b of the lactating female was slightly ($0.2^\circ C$) but significantly lower than of the non-lactating animal, but minimum T_b was much lower ($25.0^\circ C$ lactating vs $28.0^\circ C$ non-lactating) and we suggest that lactating females may conserve energy by having a labile T_b . While the mean amount of water diverted to milk was calculated as 11.5 ml day⁻¹ representing only 8% of maternal daily water flux, transfer of milk energy was estimated as representing approximately 50% of the maternal daily energy expenditure.

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Antibody response to sheep red blood cells in platypus and echidna

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A platypus and echidna, and 2 rabbits were injected, intraperitoneally, with washed sheep red blood cells. The treatment was repeated in 28 days. Blood samples were taken every 7 days, for 8 weeks. Anti- sheep red blood cell antibodies were measured using direct and indirect haemagglutination methods. Sera samples were also examined after treatment with 2-mercaptoethanol. The antibody responses elicited in the platypus and echidna were weaker than in the rabbits. In the platypus and echidna there appeared to be little apparent secondary response, the IgM-IgG switch was weak and little IgG production was elicited.