

Monotremes and marsupials: Comparative models to better understand the function of milk

1. Introduction

Lactation is the signature characteristic of mammals wherein the mother nourishes the young by secreting a nutrient-rich milk from her mammary gland. Indeed, naming of the class Mammalia by Linnaeus in 1758 (Linnaeus 1758) emphasizes lactation as the dominant characteristic for the identification of mammals, despite the existence of other anatomical characteristics. This preference reflects the fundamental influence that lactation has during the early life on all mammals and there is ample evidence that lactation has developed as a very effective and adaptable means of postnatal nutrient provision among the vertebrates during the course of evolution (Blackburn 1993).

Different reproductive strategies classify the mammals into three subclasses: the Monotremes, Marsupials and Eutherians. Monotremes and marsupials represent less than 10% of the total mammalian species, whereas the majority of mammals are grouped under the subclass Eutheria. Nevertheless, milk secretion is a common and complex process in all the mammals and it is now apparent that milk has roles that extend beyond the provision of nutrition to the suckling young. For example, milk may direct the development of organs such as the gut, can stimulate either the proliferation or apoptosis in the mammary gland and can deliver antimicrobials to protect the mammary gland and the young from infections (Berseth *et al.* 1983; Yamashiro *et al.* 1989; Wilde *et al.* 1999; Gallois *et al.* 2007). This concept can be better understood by reviewing the lactation processes in animals with extreme adaptations to reproduction. The different lactation strategies of monotremes and marsupials allow us to better unravel the role of milk proteins in the reproductive process (Brennan *et al.* 2007; Sharp *et al.* 2008; Menzies *et al.* 2009; Wang *et al.* 2011).

2. Mammals: Diversity of lactation

During evolution, amniotes (tetrapods that have a terrestrially adapted egg) split into the sauropsids (which led to reptiles and birds) and synapsids (which led to mammal-like reptiles) about 320 Mya. Monotremes emerged during the earliest split in the mammalian phylogeny as Prototherians, separated from the Theria about 166 to 220 Mya (Bininda-Emonds *et al.* 2007; Hedges and Kumar 2009). The Theria later split into the Metatherian (marsupials or Marsupialia) and Eutherian (Placentalia) lineages approximately 140 Mya. Today, after more than 200 My of evolution, the diversity of mammalian species and the extreme variations in their reproductive strategies, in particular the lactation cycles, provide numerous examples of either lineage or species-specific adaptations of the lactation system (Lefèvre *et al.* 2010). The monotremes are a fascinating combination of reptilian and mammalian characters. They retain a primitive component of reptilian reproduction, laying of shelled eggs, and this is accompanied by a prototherian lactation process, marking them as representatives of early mammals. The platypus, a semi-aquatic carnivore and two species of echidna, a terrestrial insectivore, are the only extant monotremes and are confined to Australia and New Guinea. The mothers lay small parchment eggs, which are covered by a leathery shell. The eggs are incubated outside the mother's body (inside the pouch in the case of echidna) while she secretes milk that is excreted from a series of ducts opening directly on the surface of the ventral skin patch of the areola (figure 1). Tiny hatchlings are altricial and depend completely on milk as a source of nutrition during the

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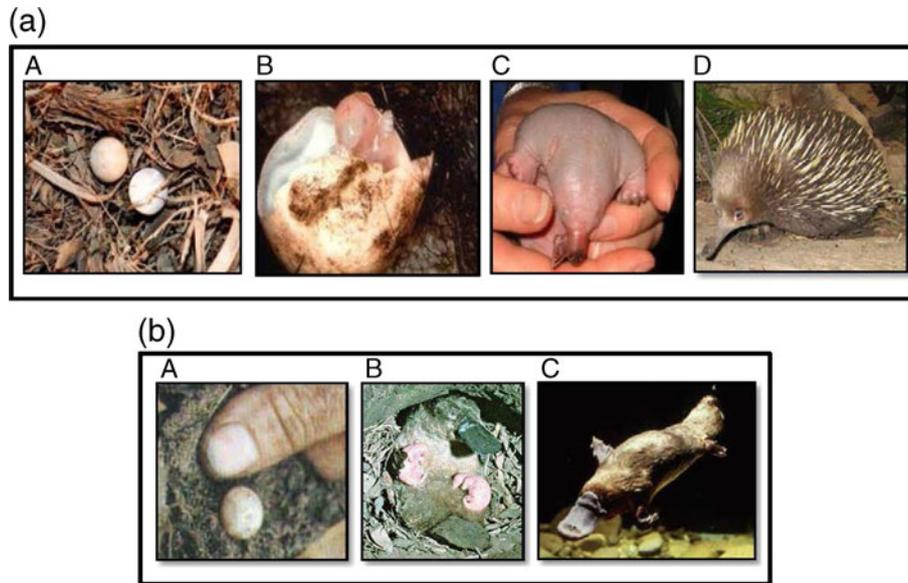


Figure 1. Monotreme neonates. (a) Developmental stages of echidna: (A) Echidna eggs; (B) Echidna puggle hatching from egg; (C) Echidna at 30 days of age; (D) Adult echidna. (b) Developmental stages of platypus: (A) Platypus egg; (B) Mother platypus suckling her young; (C) Adult platypus.

period of suckling, which is prolonged relative to gestation and incubation (Griffiths 1978). Much of the development of the monotreme young occurs after hatching and before weaning and the role of the milk in this process needs to be examined.

Marsupials have a similar reproductive strategy and present one of the most sophisticated lactation programmes yet described. After a short gestation, marsupials give birth to an altricial young that are totally dependent upon the progressive changes in milk composition for normal growth and development during the extended lactation period (Tyndale-Biscoe and Renfree 1987). By contrast, eutherians have invested in extended intrauterine development of the young and produce a milk of relatively constant composition, apart from the initial colostrum (the first lacteal secretion produced by the mammary gland in late pregnancy and immediately postpartum, prior to the production of milk). However, there are interesting adaptations to lactation within Eutherian species. For example, the three families of pinnipeds, comprising phocids (true seals), odobenids (walrus) and otariids (sea lions, fur seals), present an interesting, extreme strategy for lactation. The walrus has the lowest reproductive rate of any pinniped species and the calves accompany their mothers from birth, nursing on demand during these trips and are not weaned for at least 2 years. Phocid seals evolved large sizes to reduce heat loss and risk of predation and to increase body reserves. This enabled them to adopt a fasting strategy of lactation whereby amassed body reserves of stored nutrients facilitate fasting on land during continuous milk production over relatively short periods of 4–42 days, depending on the species (Oftedal *et al.* 1987). On the other hand, otariid seals retained smaller body sizes and insulating fur, adopting a foraging lactation strategy that is characterized by alternation between periods of several days of copious milk production on shore and extended periods of maternal foraging at sea (Bonner 1984). Evidently, lactation has played an important role in the worldwide adaptive radiation of mammals (Blackburn 1993).

3. Platform technologies to exploit diverse lactation strategies

Milk is the major source of nutrients, immunity and other major bioactives for the young in mammals (Warner *et al.* 2001). Milk also contains components that contribute to the regulation of the mammary gland of the mother (Trott *et al.* 2003; Palmer *et al.* 2006; Sharp *et al.* 2006; Topcic *et al.* 2009; Khalil *et al.* 2011; Maksimovic *et al.* 2011). However, other unknown roles played by this biological fluid remain to be

established and mammals with extreme adaptations provide new opportunities to identify regulatory molecules that are present but not readily identified in higher mammals (Sharp *et al.* 2009). To better understand the many roles of milk, we have employed different technology platforms that include genomics, proteomics and bioinformatics together with marsupials like the tammar wallaby and monotremes like echidna and platypus, which vary in their reproductive strategy from other eutherians.

3.1 *Monotremes*

The egg-laying mammals, the monotremes, are regarded as representatives of ancient mammals, occupying a very esteemed position in the mammalian tree of evolution (Lefèvre *et al.* 2010). Since the access to their milk and tissues is limited, a non-invasive approach for molecular analysis of lactation has been developed. In protected species such as the platypus (*Ornithorhynchus anatinus*) and the short-beak echidna (*Tachyglossus aculeatus*), a cDNA sequencing approach using cells harvested from milk during late lactation has proved effective (Lefèvre *et al.* 2009). This approach has provided information about a number of caseins, especially about the recent duplication of β -casein, and whey protein genes. The EST library constructed in this way now serves as a valuable source of information, for example, the identification of novel monotreme-specific bioactive proteins that have antimicrobial and proliferative activities (unpublished data). It would be intriguing to study the selective pressures responsible for the disappearance of these genes in the other mammals during the course of evolution and how the subsequent changes relate to changed reproductive strategies.

The milk that is secreted just prior to hatching of the eggs in monotremes is speculated to protect the eggs (Ofstedal 2002) and the subsequent altricial hatchlings from microbial infections. It is reasonable to predict the presence of various bioactives in milk since much of the development of monotreme young occurs in the non-sterile environment. We have identified a novel monotreme-specific protein of 90 amino acids, expressed during lactation, and our preliminary data have validated its antibacterial activity against a host of gram-positive and gram-negative bacteria. This approach is being expanded to identify bioactive proteins secreted in the milk using the SST-REX method (signal-sequence trap by retroviral mediated expression screening). This method was developed by Kojima and Kitamura (1999) and detects signal sequences in cDNA fragments based on their ability to redirect a constitutively active mutant of a cytokine receptor to the cell surface, thereby permitting interleukin-3 (IL-3)-independent growth of murine Ba/F3 cells (Kojima and Kitamura 1999). This library of genes coding for secretory proteins of monotreme milk cells provides valuable data to interpret aspects of milk secretion from an evolutionary perspective as we progress up the mammalian tree.

3.2 *Marsupials*

The tammar wallaby (*Macropus eugenii*), an Australian marsupial, has a reproductive strategy which is different from other mammals. The tammar has a short gestation period of 26.5 days and major development of the altricial young occurs postpartum. Development of the young is regulated by the mother's milk, which progressively varies in composition across the extended 300 days of the lactation cycle (Tyndale-Biscoe and Janssens 1988; Nicholas *et al.* 1997) (figure 2). This adaptation in reproductive strategy provides new opportunities for understanding the role of different milk proteins both for the development of the young and the function of the mammary gland.

A cDNA library had been produced from mammary tissues of tammar wallaby at different stages of the lactation cycle (Lefèvre *et al.* 2007). This has generated around 14,837 expressed sequence tags, which represent about 25% of the tammar genome. This is one of the largest cDNA resources from a marsupial and has about 10% marsupial-specific mammary transcripts, whereas 15% are mammal-specific. A database has been established as a resource to identify either genes or proteins having role in providing nutrition and immunity to the young, and those having a role in development and providing protection to mother's mammary gland. About 5000 mammary genes have been transcript profiled during lactation using a tammar EST microarray, and at least 75 novel secretory proteins were identified (Sharp *et al.* 2009).

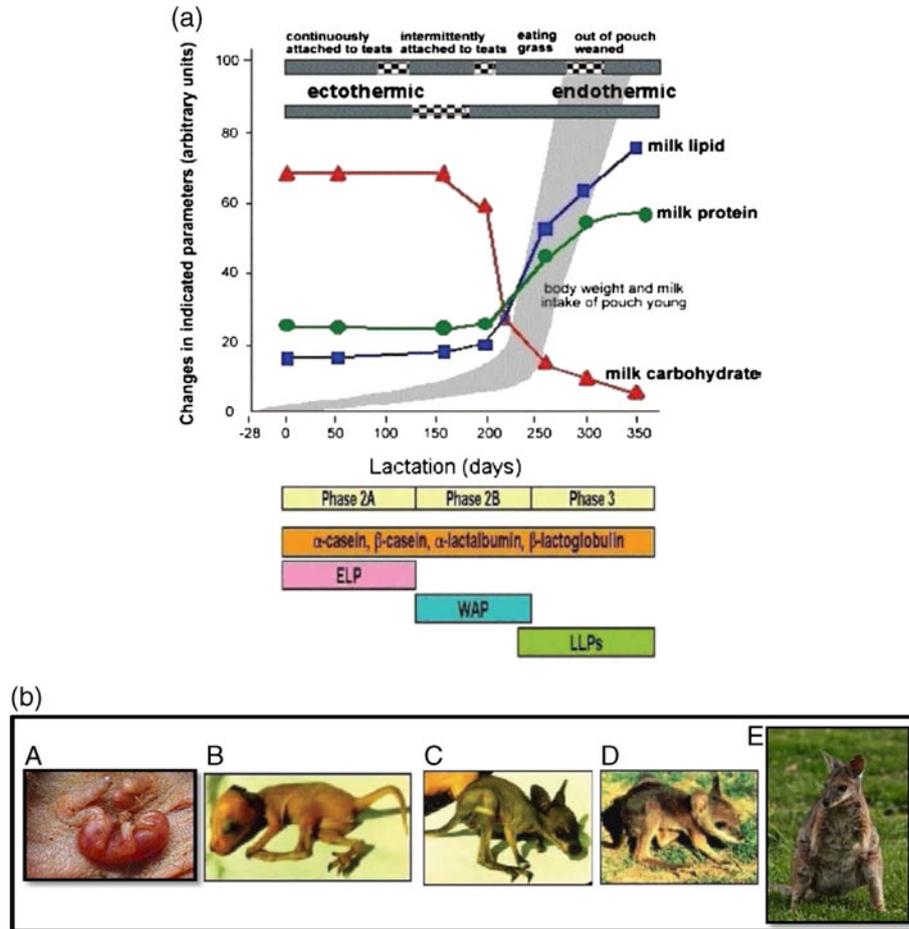


Figure 2. Lactation cycle of tammar wallaby. (a) Phases of the lactation cycle of tammar wallaby with major milk protein genes α -casein, β -casein, α -lactalbumin and β -lactoglobulin expressed throughout lactation and other genes ELP (early lactation protein), WAP (whey acidic protein), LLP (late lactation protein A and B) expressed at specific phases of lactation. (b) Developmental stages of tammar wallaby: (A) Day 6; (B) Day 70; (C) Day 185; (D) Day 220; (E) Adult.

The lactation strategy of tammar offers a good model system to study the role of milk in the development and protection of the mother and pouch young. One of the major protein groups differentially expressed across the lactation cycle are the cathelicidins, proteins known to have antimicrobial (Zanetti *et al.* 1995; Yang *et al.* 2004) and proliferative activity (Heilborn *et al.* 2003; Shaykhiev *et al.* 2005). In the tammar, cathelicidins were first identified and characterized by Daly *et al.* (2008), who proposed the protein to play a role in providing innate immunity to the pouch young based on the high level of expression during the initial stages of development. Recently Wanyonyi *et al.* (2011) studied two major cathelicidin genes expressed in the mammary gland of the tammar wallaby. MaeuCath1 and MaeuCath7, the full-length peptides encoded by these genes, showed antimicrobial and cell proliferative activity. MaeuCath1 exhibited alternate splicing, with MaeuCath1a and MaeuCath1b variants showing differential expression across lactation. The MaeuCath1a variant showed antibacterial activity and the gene was highly expressed during initial stages of development of the young, and Wanyonyi *et al.* (2011) proposed a role for this protein to provide immunity for the immune-incompetent pouch young. Expression of this gene was again increased at involution, suggesting that MaeuCath1a may provide protection to the involuting mammary gland at a time when it is susceptible to increased infection (Oliver and Mitchell 1983). The second splice variant MaeuCath1b showed proliferative activity on mammary epithelial cells and was up-regulated during mid and late lactation when there was maximal growth of the mammary gland and milk production. Thus, the tammar cathelicidin gene is temporally regulated by alternate splicing to provide protection for both pouch

young and the mother's mammary gland during high risk of infection and to help proliferation of mammary gland during maximal growth of the gland.

The WFDC2 protein [whey acidic protein (WAP) four disulfide core proteins] is another antimicrobial protein whose expression in the mammary gland of tammar wallaby was recently identified (Watt *et al.* 2012). The tammar WFDC2 protein has two domains: the amino terminal end is domain III and the carboxy terminal end is domain II (Sharp *et al.* 2007). Domain II of this protein had antibacterial activity and this property was strain-specific with no activity against gut commensal *Enterococcus faecalis* (Watt *et al.* 2012). This protein was up-regulated during pregnancy, early lactation and involution, which correlated with the stages during which the altricial young is totally dependent on milk for immune protection and later on when the mammary gland is more susceptible to infection. Thus, the reports on cathelicidins and WFDC2 shows the importance of a marsupial model to study the role of milk in providing timely immune protection to the pouch young and the mother's mammary gland and also in playing a role in development of the mammary gland.

Whey acidic protein (WAP), a family of the 4-DSC (four disulphide core) domain proteins, is a major whey protein whose function is not well understood in many species. This protein has been identified in milk throughout lactation in a number of eutherians including mouse, rat, (Hennighausen and Sippel 1982; Campbell *et al.* 1984) rabbit (Devinoy *et al.* 1988) camel (Beg *et al.* 1986) and pig (Simpson *et al.* 1998). There is loss of WAP functionality in human and ruminants like cow, goat and ewe, where there is existence of a pseudo-gene due to a frame shift mutation in ruminants (Hajjoubi *et al.* 2006) and a coding mutation in human (Rival-Gervier *et al.* 2003). WAP is also secreted in the milk of many marsupials, including the tammar wallaby, where the expression is specific to mid lactation (Simpson *et al.* 2000). This phase-specific expression of WAP in the tammar wallaby may be considered a phase-specific knock-out model and provides new opportunities in understanding the function and reasons for its absence in other

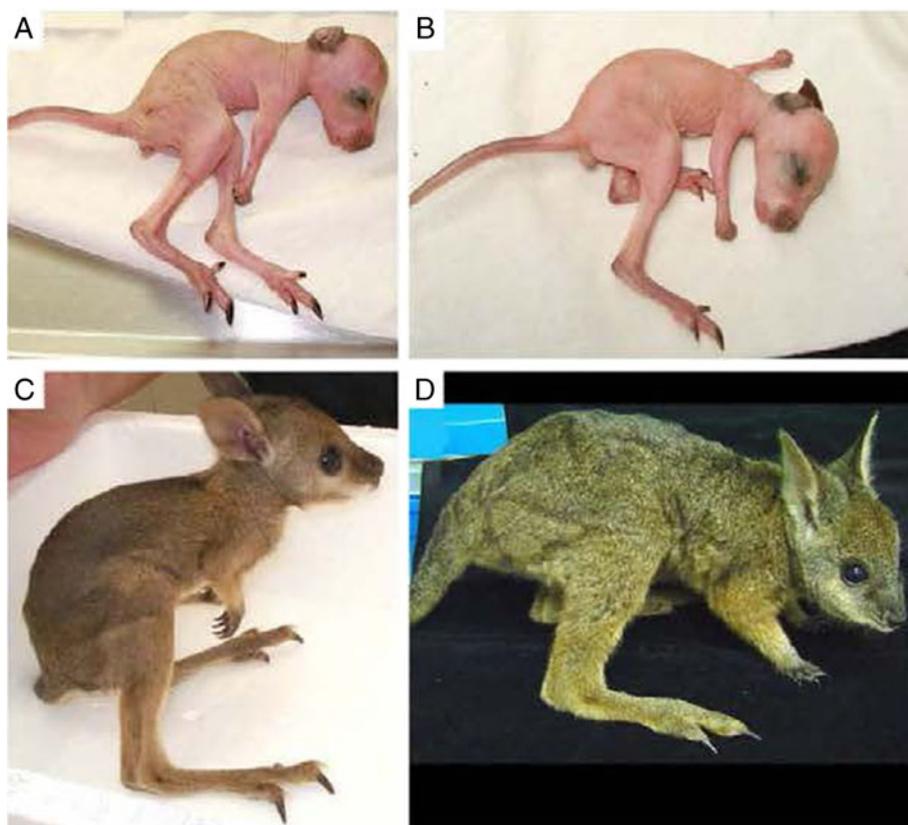


Figure 3. Cross-fostering experiment in tammar wallaby. (A) and (B) are PY of 120 days used in control and fostered group. (C) and (D) are after 60 days of control and fostered group

species. Marsupial WAPs are characterized by the presence of three domains (I, II and III) (Simpson *et al.* 2000) and the domain III specific to tammar was responsible for the proliferative property on mammary epithelial cells (MEC) (Topcic *et al.* 2009). In contrast, domains I and II, which were more comparable to the eutherian WAP, did not have any activity on MEC. This altered function for a single protein in eutherians and marsupials may be due to the difference in the functional domain III and be correlated with the different reproductive strategies between marsupials and eutherians.

The tammar wallaby exhibits another unique mechanism called concurrent asynchronous lactation (CAL) whereby a new born pouch young and an older animal out of the pouch, each receives milk of different composition from adjacent mammary glands simultaneously. This suggests that the two mammary glands are functioning independently and each gland is controlled locally (Nicholas *et al.* 1997). This mechanism is consistent with the role of milk in regulating growth and development of the pouch young. In addition to these nutritional and developmental factors, there is high probability that tammar milk may contain negative factors like the feedback inhibitor of lactation, which is involved in preventing milk secretion (Hendry *et al.* 1998).

Apart from proteins playing a role in development and protection of mother's mammary gland and infant, there are specific bioactives in milk that signal development of specific tissues. This has been demonstrated in the tammar by employing a cross-fostering strategy in which a younger pouch young was fostered to a mother secreting later-stage milk and this transfer resulted in an accelerated development of the young (Trott *et al.* 2003; Waite *et al.* 2005; Kwek *et al.* 2009) (figure 3). It was further demonstrated by a similar strategy that later-stage milk activated stages of stomach development in the pouch young (Kwek *et al.* 2009). Therefore, using this marsupial model where the composition of milk varies across lactation, we can identify different potential factors in milk that may play a role in specific tissue development.

It is known that in eutherians including humans, the early growth and development of the foetus is regulated *in utero*. Preterm babies and babies born small for age have a higher risk of mortality, and if their growth is accelerated outside the uterus, there are subsequent chronic health problems generally termed mature onset disease (MOD). These mainly include type 2 diabetes, retinopathy of prematurity, bacteremia and hypertension (Newsome *et al.* 2003; Leviton *et al.* 2010). In marsupials such as the tammar, where the mother gives birth to an altricial young, it is apparent that the growth and development is dependent on the bioactives in milk at different stages of lactation. Thus, the tammar can be utilized as a model in identifying emerging roles of milk in both development of the young and the mother's mammary gland. These studies may provide a better understanding of the timing of signals delivered to the young to develop strategies to improve survival of young with low birth weight and subsequent treatment of MOD.

4. Conclusion

There are a vast number of milk proteins that are not characterized in mammals. Monotremes and marsupials have diverse reproductive strategies where the roles of milk are more apparent than in eutherians, and they are, therefore, proving to be legitimate biomedical models for identifying and characterizing these bioactives. Different technology platforms like genomics, proteomics and bioinformatics can be utilized to exploit these model species to better understand the role of milk in development and protection of both mother and infant. Ultimately, as Capuco and Akers (2009) said, '*Because no single species can provide an ample and sufficient model for the physiology of another, and because the potential gain in knowledge from comparative studies is great, the research community should not be species-centric*'.

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