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The evolution of milk secretion and its ancient origins

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Lactation represents an important element of the life history strategies of all mammals, whether monotreme, marsupial, or eutherian. Milk originated as a glandular skin secretion in synapsids (the lineage ancestral to mammals), perhaps as early as the Pennsylvanian period, that is, approximately 310 million years ago (mya). Early synapsids laid eggs with parchment-like shells intolerant of desiccation and apparently dependent on glandular skin secretions for moisture. Mammary glands probably evolved from apocrine-like glands that combined multiple modes of secretion and developed in association with hair follicles. Comparative analyses of the evolutionary origin of milk constituents support a scenario in which these secretions evolved into a nutrient-rich milk long before mammals arose. A variety of antimicrobial and secretory constituents were co-opted into novel roles related to nutrition of the young. Secretory calcium-binding phosphoproteins may originally have had a role in calcium delivery to eggs; however, by evolving into large, complex casein micelles, they took on an important role in transport of amino acids, calcium and phosphorus. Several proteins involved in immunity, including an ancestral butyrophilin and xanthine oxidoreductase, were incorporated into a novel membrane-bound lipid droplet (the milk fat globule) that became a primary mode of energy transfer. An ancestral c-lysozyme lost its lytic functions in favor of a role as α-lactalbumin, which modifies a galactosyltransferase to recognize glucose as an acceptor, leading to the synthesis of novel milk sugars, of which free oligosaccharides may have predated free lactose. An ancestral lipocalin and an ancestral whey acidic protein four-disulphide core protein apparently lost their original transport and antimicrobial functions when they became the whey proteins β-lactoglobulin and whey acidic protein, which with α-lactalbumin provide limiting sulfur amino acids to the young. By the late Triassic period (ca 210 mya), mammaliaforms (mammalian ancestors) were endothermic (requiring fluid to replace incubatory water losses of eggs), very small in size (making large eggs impossible), and had rapid growth and limited tooth replacement (indicating delayed onset of feeding and reliance on milk). Thus, milk had already supplanted egg yolk as the primary nutrient source, and by the Jurassic period (ca 170 mya) vitellogenin genes were being lost. All primary milk constituents evolved before the appearance of mammals, and some constituents may have origins that predate the split of the synapsids from sauropsids (the lineage leading to 'reptiles' and birds). Thus, the modern dairy industry is built upon a very old foundation, the cornerstones of which were laid even before dinosaurs ruled the earth in the Jurassic and Cretaceous periods.

Keywords: evolution, lactation, milk composition, casein, milk fat globule

Implications

Lactation has an evolutionary origin and biological context within which its secretory processes and milk constituents evolved. This review provides an evolutionary scenario within which the specific functions of milk constituents can be investigated. It is hoped that this review will stimulate increased attention to the evolution of lactation and milk constituents in a large variety of disciplines, including molecular biology, evo-devo, comparative genetics, protein structural studies, dairy chemistry, paleobiology, phylogenetic systematics, mammary gland biology, reproductive evolution, and comparative nutrition. The goal is an in-depth understanding of how the remarkable process of lactation came into being.

Introduction: the importance of milk to mammals

Milk secretion is a characteristic feature of all mammalian species, large or small, social or solitary, arctic or tropical. The famous taxonomist Carolus Linnaeus selected the name Mammalia in 1758 in uniting terrestrial 'quadrupeds' with dolphins and whales to reflect the fact that females of both groups bear mammae, or mammary glands (Gregory, 1910). Lactation is unique to mammals. No other extant organism produces copious glandular skin secretions to feed its young,

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although the larvae of some fish ingest modest amounts of nutritive mucus from surface mucous glands (Buckley *et al*., 2010); terrestrial-breeding frogs provide water, antimicrobial compounds, and perhaps some nutrients to their eggs via granular and/or mucous gland secretions (Taigen *et al*., 1984; Oftedal, 2002a); and the young of some live-bearing caecilians feed on sloughed skin, skin secretions, or perhaps both (Kupfer *et al*., 2006). The 'crop milk' of pigeons, flamingoes and emperor penguins is a lipid-rich material produced by holocrine secretion by epithelial cells of the esophagus or crop (Horseman and Buntin, 1995), but it does not match milk in complexity, magnitude or duration of secretion. Lactation can persist for many years in humans, great apes, toothed whales, and elephants (West *et al*., 2007), but may be as little as 3–4 days in the ice-breeding hooded seal (Oftedal *et al*., 1993a).

Lactation is highly complex and apparently of ancient evolutionary origin (Oftedal, 2002b; Lefevre *et al*., 2010). The detailed signaling crosstalk among epithelial and underlying mesenchyme cells that is required for the differentiation, ductal branching, and proliferation of mammary tissue is only now being unraveled (Watson and Khaled, 2008), and the functional significance and patterns of expression of thousands of mammary genes are under investigation (Lemay *et al*., 2009). Secreted milk is extremely varied in composition – from trace levels of fat in rhinos to more than 60% fat in some ice-breeding seals (Oftedal and Iverson, 1995) – and contains unique proteins (α-, β-, and κ-caseins, β-lactoglobulin, α-lactalbumin, whey acidic protein (WAP)), membrane-enclosed lipid droplets, and sugars (lactose, milk oligosaccharides) that are not found elsewhere in nature.

A dependency on milk is key to the life history strategy of every mammal. Many mammals specialize on diets that would be too difficult for small offspring to capture or to digest, or that might fail to cover the nutrient needs of rapidly growing young if milk was not available (Pond, 1977). The ability to provide offspring with a highly digestible, nutritionally balanced, and variably concentrated food has enabled mammals to evolve a wide range of developmental and reproductive strategies. Lactation provides the essential nutrients required by the smallest and most altricial of neonates, such as monotreme hatchlings and marsupial neonates (Griffiths, 1978; Tyndale-Biscoe and Renfree, 1987), as well as the largest and most precocial of offspring, such as hooded seals that only nurse for 4 days, but in this time double their mass (Oftedal *et al*., 1993a). Milk production can be miniscule, as it must have been to feed newborn Tasmanian tigers (or thylacines) which, by extrapolation from living dasyuromorph marsupials, weighed less than 40 mg at birth, equivalent to 0.00015% of the mass of the 25 kg mother, surely the smallest birth mass : maternal mass ratio of any mammal (O. Oftedal, unpublished results). However, milk production can also be immense, as in the 100 000 kg blue whale. The pregnant female feeds in polar and cold temperate waters where food is abundant and where she lays down massive stores of blubber. She then travels part way around the globe to warm or tropical areas, gives birth to a calf, and while fasting or feeding a little, produces an estimated 220 kg milk per day, containing approximately 4000 MJ energy (Oftedal, 1997). Over the course of the 6-month lactation, the blue whale cow transfers an estimated 700 000 MJ to her calf, enough energy to feed approximately 200 people for a year.

The ability to lactate allows mothers to collect nutrients and deposit them in their tissues at one time and location, but then provide these nutrients to their young as milk at a later time and/or another location, such as in a nest or burrow (rodents), cave (bats, bears) or snow den (polar bear) or on an isolated island (sea lion, fur seal) or on floating rafts of pack ice (various seals, walrus; Oftedal, 2000). Some bears lactate in the winter den for 2 months before emerging to feed and drink, a feat made possible by mobilization of extensive body reserves, production of milk low in sugar (minimizing the glucose demand of the mammary glands), and recycling of water and perhaps nitrogen from cubs back to the mother when she ingests their excreta (Oftedal *et al*., 1993b). In bats, lactation must continue until pups have deposited enough protein and mineral that their muscles and bones have sufficient strength to withstand the pressure and torque associated with flight, or the pups cannot feed themselves; bat pups may individually reach 70% of maternal mass before they are weaned (Hood *et al*., 2011). In social carnivores, an entire pack may hunt prey that is brought back to the lactating female, who converts food constituents into mammary secretions. The lactating female is in essence a 'milk factory', rearing very large litters of rapidly growing young (Oftedal and Gittleman, 1989).

How did such a remarkable fluid as milk and such remarkable biochemical factories as mammary glands evolve? The mammary gland and its secretion represent major evolutionary novelties, without any known intermediates. In the mid-19th century the complexity and interdependence among mammary glands, milk and dependent suckling young posed a challenge to Charles Darwin's theory of evolution by natural selection. Darwin rose to the challenge, devoting most of a chapter of the 1872 edition of *On the Origin of Species* to a discussion of the problems of evolutionary novelty, such as the origin of the eye and of the mammary gland. Believing that seahorse eggs are hatched and reared in a brood pouch, Darwin (1872: pp. 295–296) suggested by analogy that if mammalian young were protected in a pouch containing cutaneous glands, a gradual increase in the nutritive quality of the fluid and of the secretory complexity of the glands would be favored by natural selection, leading ultimately to breasts producing milk. Over the years since, a variety of authors have speculated on the origin and evolution of lactation, trying to envision a process in which something so complex could have evolved step by step and be favored by natural selection (reviewed by Oftedal, 2002b). Unfortunately, most of these scenarios (including a recent suggestion that lactation originated from the innate immune system (Vorbach *et al*., 2006; McClellan *et al*., 2008)) have been put forth without reference to an evolutionary timescale and do not acknowledge the repeated radiations of increasingly mammal-like taxa since the first appearance of synapsids approximately 310 million years ago (mya).

Milk secretion in the context of synapsid evolution

The phylogenetic branch that would ultimately lead to mammals (Synapsida) first diverged from the branch leading to 'reptiles' and birds (Sauropsida) in the mid-Pennsylvanian period, approximately 310 mya. After this separation, there were a series of sequential extinctions, with only a limited number of taxa surviving into the succeeding geologic periods as the basis for future radiations (Sidor and Hopson, 1998; Oftedal, 2002b; Kemp, 2005). Thus, the basal synapsids radiated in the Pennsylvanian period and the early Permian period; however, most lineages went extinct by the mid–late Permian period, when they were succeeded by a radiation of therapsids. Most therapsid taxa disappeared during a massive extinction event at the end of the Permian period, but cynodonts survived to radiate in the Triassic period. Most of these lineages in turn disappeared in the late Triassic period, but a subset – the mammaliaforms – radiated in the late Triassic and Jurassic periods. It was from within the mammaliaforms that true mammals evolved, perhaps in the late Jurassic period, approximately 160 mya.

These sequential radiations incorporated an increasing number of anatomic traits that now characterize mammals, that is, they became progressively more mammal-like (Sidor and Hopson, 1998; Kemp, 2005). Some of these traits involved changes in locomotion (from a sprawled lizard-like gait to an upright stance with improved running ability), in growth pattern (from a periodic pattern of bone mineralization to more continuous growth), in respiratory ability (development of diaphragmatic breathing), in heat exchange (using respiratory surfaces in the nasal cavity for cooling and moisture retention) and in food processing ability (rearrangement of skull and jaw bones for increased jaw musculature, diversification and specialization of teeth and tooth cusps). The picture is one of increasing metabolic expenditure, increased growth rates, increased activity, and a presumed upregulation of basal metabolism and increased refinement of temperature regulation associated with endothermy.

Reproductive patterns also changed. The early synapsids were egg-laying, like sauropsids (the lineage leading to 'reptiles' and birds) (Oftedal, 2002a). It was the development of a complex of extraembryonic membranes to facilitate respiration, use of stored yolk nutrients and segregation of waste products within the egg that allowed the amniotes (including sauropsids and synapsids) to venture onto land and to gain a measure of independence from free water (Packard and Seymour, 1997). However, unlike sauropsids, the synapsids never evolved a calcified eggshell, or at least no fossil record of such has ever been discovered (in contrast to the plethora of fossilized eggshells from dinosaurs, crocodilians, turtles, birds and other sauropsids; Oftedal, 2002a). An egg with a parchment-like shell is the ancestral condition of both groups; however, most sauropsids went down a different evolutionary path, developing elaborate eggshells of diverse crystalline form. These calcified eggshells are very resistant to vapor loss, having only small pores for gas exchange. By contrast, the eggs with parchment-like shells both gain and lose water rapidly. Eggs with parchment-like shells are ectohydric, relying on environmental water for completion of egg development, whereas eggs with calcified shells are by necessity endohydric, relying on water invested in the egg before calcification and laying. Eggs with parchment-like shells dry out rapidly when exposed to a vapor pressure gradient, as in air that is not fully saturated with moisture, or when exposed to a positive temperature gradient from egg to environment (Oftedal, 2002a).

Early synapsids must either have buried their eggs in moisture-laden soil, as do living lizards and snakes that produce eggs with parchment-like shells, kept them hydrated by contact with moist skin (as terrestrial-breeding frogs and salamanders often do) and/or retained them within a moist pouch, such as the egg-laying echidnas in Australasia. Yet during the long period from the Pennsylvanian period to the Jurassic period, the therapsids, cynodonts and mammaliaforms are believed to have increased metabolic rates and elevated body temperatures, and eggs were presumably incubated to increase thermal stability (Oftedal, 2002a). A buried egg has access to soil moisture, but is at soil temperature, whereas an incubated egg in a nest is both isolated from soil moisture and – due to thermal gradients that create vapor pressure gradients – subject to rapid desiccation. Pythons that incubate eggs with parchment-like shells above ambient temperatures wrap tight body coils about the eggs to restrict moisture loss (Lourdais *et al.*, 2007). Some lizards and snakes overcome this constraint by internal egg-retention, including development of placental structures to provide nutrients to the developing embryos (Thompson *et al.*, 2006). However, early mammals were still egg-laying (as indicated by egg incubation in extant monotremes and the persistence of egg teeth in some marsupial neonates; Oftedal, 2002a and 2002b).

It is not clear why the synapsids lineage did not evolve egg-retention as a reproductive strategy, as this occurred independently many times among sauropsids (Blackburn, 2006). Perhaps some radiations did, but went extinct without leaving descendants. The synapsids that ultimately survived to become mammaliaforms and mammals opted for a different course: reduction in egg size and a switch to lactation as the primary nutrient source for offspring.

Yet it seems impossible that the increasingly endothermic synapsids could have incubated their eggs without facing lethal egg dehydration unless there was an exogenous source of moisture. I have argued that this required the evolution of lactation, initially as a source of moisture for eggs (Oftedal, 2002a). Early synapsids apparently had glandular skin, and like extant frogs and salamanders that rear terrestrial eggs, they may have kept eggs moist via skin secretions (see 'The evolution of mammary secretion' section). As eggs and hatchlings were in contact with skin secretions rich in antimicrobial and other protective compounds (see Whey Proteins section, below), the evolution of nutrient-rich constituents in these secretions could occur gradually and over a long time period. Thus, milk was born.

It is not known whether eggs and hatchlings were kept in a pouch, as Darwin had hypothesized, but this would have

been an effective means of reducing moisture loss by eggs and hatchlings. Many paleontologists have considered epipubic bones as evidence that mammaliaforms and early mammals (including early eutherians) carried eggs or young in a pouch or suspended from the abdomen; however, others argue that epipubic bones rather had a function in locomotion as the structure of the pelvis evolved (Novacek *et al.*, 1997; Reilly and White, 2003). Pouches have evolved in both monotremes (e.g. the echidnas) and marsupials (e.g. kangaroos and wallabies), but these may not be of ancestral origin; pouches appear to have been gained and lost multiple times in marsupials (Tyndale-Biscoe and Renfree, 1987).

During the Triassic and the early Jurassic periods, the cynodonts and mammaliaforms were not only developing elevated metabolic rates but also were becoming progressively smaller in size (Kemp, 2005). This miniaturization of body size would have required miniaturization of eggs as well. Even if eggs were kept in a pouch to prevent desiccation, once they hatched the young would be too small to be effective homeotherms (Hopson, 1973). In birds, reduction of egg size in small species is accompanied by the reduction of incubation time and hatching of altricial (incompletely developed) young (Starck and Ricklefs, 1998). Hopson (1973) argued that the diminutive mammaliaforms (some no more than a few grams as adults; Luo *et al.*, 2001) must have been producing altricial young, as small eggs could not hold enough yolk to allow development of precocial (well developed) hatchlings. However, altricial young would require feeding, indicating that lactation had already evolved.

This conclusion is bolstered by the fact that late cynodonts and the mammaliaforms developed a reduction in tooth replacement (Kemp, 2005). Early synapsids, like most sauropsids, had teeth that were replaced continuously as the animal grew, allowing smaller teeth to be replaced by larger teeth as the jaw lengthened with age. Most mammals, by contrast, have only two sets of teeth, an initial set of deciduous 'milk teeth' and the adult dentition. This developmental strategy, termed diphyodonty, is possible because tooth eruption is delayed as the jaw develops *in utero* and during the lactation period. There is no need for a robust, adult-type teeth in dependent offspring that do not need to capture or consume an adult-type diet. The fact that late cynodonts and early mammaliaforms already had evolved diphyodonty indicates that they were already reliant on milk (Oftedal, 2002b).

I conclude that the sequential radiations of basal synapsids, therapsids, cynodonts and mammaliaforms from the Pennsylvanian period through the Jurassic period (i.e. from approximately 310 to 160 mya) was a time of increasing reliance on glandular skin secretions, to keep skin moist and pliable, to provide water to eggs, and finally as a nutrient source for offspring.

The evolution of mammary secretion

The mammary gland is a complex alveolar–ductal organ involving a layer of secretory epithelial cells (lactocytes) that secrete milk into the alveolus by a variety of mechanisms (including exocytosis of secretory vesicles and budding out and pinching off of milk fat globules, MFGs), a surrounding layer of myoepithelial cells that expel milk from the alveolus via contraction, and a basement membrane that surrounds the epithelial compartment. The many alveoli are connected to the skin surface by a much-branched ductal network that terminates in canals, or galactophores, that penetrate either thickened epithelial structures (the nipples) in eutherians and marsupials or a specialized mammary patch or areola in monotremes (Oftedal, 2002b).

The entire structure initiates development early in embryonic life (before hatching from the egg in monotremes) via coordinated reciprocal signaling between the epithelial cells of ectodermal origin and the underlying mesenchyme of mesodermal origin. This signaling directs gene expression, morphogenesis of tissue architecture, and differentiation of tissue-specific functions (Robinson, 2004; Watson and Khaled, 2008). For example, signaling compounds generated by the mesenchyme/stroma that have receptors in the epithelial cells and that help determine ductal and alveolar development include hepatocyte growth factor (HGF), IGF-1, activin/inhibin B, epimorphin, neuroregulin and keratinocyte growth factor (also called fibroblast growth factor-7; Nelson and Bissell, 2006). This type of branching morphogenesis driven by epithelial–mesenchymal interactions and involving coordinated development with stimulatory signaling in part from HGF and epidermal growth factor, balanced by inhibitory signaling from members of the transforming growth factor-β family, is also found in tissues of even more ancient evolutionary origin, such as the pancreas, lung, kidney, prostate and salivary glands (Nelson and Bissell, 2006). Thus, the developmental pathways of the mammary gland must derive from some pre-existing tissue, presumably glandular tissue associated with the skin, which was co-opted for a new function, the secretion of a nutritive fluid for feeding of the young. Although the basic pattern of mammary development, and its regulation, may derive from a more ancient model, the extent of glandular proliferation and output – the remarkable repeated cycles of proliferation and secretion followed by cellular apoptosis and gland involution – and the types of secretory products formed represent evolutionary novelties.

The synapsid lineage that led to mammals derived from pre-amniotic tetrapods, a group now represented by living amphibians (termed lissamphibia by paleontologists). Amphibian skin is characterized by a relatively thin epidermis containing a few cell layers of stratum corneum and dense coverage of small multicellular secretory glands that have evolved to secrete primarily mucus (mucous glands) or bioactive constituents (granular glands) onto the skin surface (Clarke, 1997). Both gland types apparently secrete via exocytosis of secretory vesicles; however, secretory cells in granular glands also swell with contents and release bulk material into the lumina of the glands via a holocrine process. This material may then be discharged onto the skin surface by contraction of myoepithelial cells when the animal is

agitated or stressed. Synapsids apparently inherited a glandular skin from the tetrapods. A remarkable early Permian fossil of the integument of the therapsid *Estemmosuchus* (Dinocephalidae) includes a dense pattern of concave lens-like structures; Chudinov (1968) interpreted these as multicellular, flask-shaped alveolar glands – similar to the glands of amphibian skin – and argued that a glandular skin is a primitive synapsid feature still evident in mammals. It should also be recognized that the complex combinations of α- and β-keratins, multi-layered scales and paucity of skin glands in living reptiles evolved after the separation of sauropsids and synapsids and is not ancestral to the mammalian integument (Dhouailly, 2009).

However, could such glands evolve into mammary glands? On the basis of evidence that elements of the innate immune system are incorporated into milk constituents and that certain signaling pathways of the innate immune system also have a role in regulating mammary development, Vorbach *et al*. (2006) speculated that mammary secretion first developed as part of an inflammatory response by mucous secreting cells, and McClellan *et al*. (2008) supported this view. The innate immune system certainly has an ancient origin, including the antimicrobial compounds associated with epithelial structures and secretions (Beutler, 2004). In frogs and other amphibians, mucous glands are important to keep the skin surface moist, facilitating exchange of respiratory gases across the very thin epidermis (Lillywhite, 2006), and amphibian cutaneous glands are indeed veritable factories of antimicrobial constituents. At least 500 antimicrobial peptides have been found in amphibian skin glands to date (Jenssen *et al*., 2006). However, mucous and granular glands are very different in structure and secretion from mammary glands. In mammals, mucous glands are restricted to oral and internal epithelial surfaces. Although Vorbach *et al*. (2006) are certainly correct that mammary glands must derive, ultimately, from the simple glandular skin structures found in pre-amniotic tetrapods (Quagliata *et al*., 2006), there must have been intermediate stages that more closely resemble mammary glands.

If one compares mammary glands to other mammalian skin glands, they bear close resemblance to only one type of gland, the apocrine glands on the general skin surface of mammals (Oftedal, 2002b). Apocrine glands and mammary glands secrete constituents both by exocytosis of secretory vesicles and by a budding out and pinching off of cellular contents with loss of cytoplasm. In apocrine glands, the latter process is considered an apocrine mode of secretion, in contrast to merocrine secretion employing exocytosis of vesicles or holocrine secretion in which cells swell with the secretory product that is released via apoptotic disruption of cellular integrity. In mammary glands, budding out and pinching off occur during secretion of MFG; cytoplasmic crescents may be present but are minimal (Mather and Keenan, 1998; Mather, 2011). It is likely that MFG secretion is a highly derived form of apocrine secretion in which upregulation of milk fat secretion has required incorporation of novel membrane constituents (see Milk Fat Globule section, below). Unfortunately, little is known about the details of secretion in generalized apocrine glands or about the genes expressed and proteins synthesized (Oftedal, 2002b), although at least some types do not produce milk-specific proteins, such as β-casein (Gritli-Linde *et al*., 2007). From an evolutionary perspective, it would be very interesting to compare the array of genes expressed by developing and secreting apocrine glands with those expressed during mammary gland development, milk secretion, and mammary involution. For example, nearly 200 milk protein genes and more than 6000 other genes have been identified as expressed in the mammary glands in virgin, pregnant, lactating, involuting, and mastitic cows (Lemay *et al*., 2009), but how many of these genes are expressed in apocrine glands is unknown. It would also be instructive to compare gene expression of both apocrine and mammary glands with that occurring in amphibian skin glands to document similarities and differences that may indicate ancestral and derived conditions.

In most mammals, an apocrine gland on the general skin surface is typically associated with both a hair follicle and a sebaceous gland in a triad termed an apo-pilo-sebaceous unit (APSU). The development of the APSU occurs in coordinated fashion, no doubt because of crosstalk between the differentiating epithelial cells and underlying mesenchyme, as well as differences in signaling pathways and receptors of the hair follicle, apocrine gland, and sebaceous gland (Hatsell and Cowin, 2006; Andrechek *et al*., 2008; Mayer *et al*., 2008). The apocrine gland duct typically opens into the infundibulum of the hair follicle, such that secretion contacts the hair shaft. Remarkably, in monotremes, a similar relationship exists between mammary glands, hair follicles, and sebaceous glands; the three form what can be termed a mammo-pilo-sebaceous unit (MPSU; Oftedal, 2002b). The galactophores (lactiferous ducts) also open up into the infundibula of enlarged, specialized mammary hairs (Griffiths, 1978). The mammary glands in monotremes are organized into a small oval mammary patch or areola consisting of 100 to 200 MPSUs (Griffiths, 1978; Oftedal, 2002b); there is no nipple. In the area surrounding the mammary patch, APSUs develop. The mature, lobular mammary gland in mid-to-late lactation is very much larger, more branched, and contains many more secretory epithelial cells than an apocrine gland, but in earliest lactation — when monotreme eggs are incubated and hatched – the mammary gland is still relatively small and tubular (Griffiths, 1978), and thus has a superficial resemblance to an apocrine gland.

In marsupials, such as opossums and kangaroos, there is also a developmental association of mammary glands with hair follicles and sebaceous glands. According to an early work by Bresslau (1912 and 1920), an oval primary-primordium separates into nipple primordia, which deepen into knobs and bud out into hair follicles (primary sprout), mammary glands (secondary sprout) and sebaceous glands (tertiary sprouts). In the opossum, for example, eight hair follicle sprouts are associated with eight mammary sprouts and eight sebaceous sprouts; that is, the nipple primordium develops into eight MPSUs. The hair follicles penetrate the nipple epithelium during development, but are subsequently

shed, each leaving a duct (galactophore) by which the mammary gland communicates to the surface of the nipple. As opossums have a dozen or more nipples, approximately 100 MPSUs are involved. In the adult marsupial, the 'mammary hairs' are no longer evident, but the galactophores bear testimony to their previous existence. Among different species, the number of ducts penetrating the nipple reflects the numbers of primary hair follicle buds, and can vary from 3 to 33 (Tyndale-Biscoe and Renfree, 1987); however, in species with large numbers of presumptive MPSUs, it is not certain that all secondary sprouts develop into functional mammary lobules.

In eutherian mammals, the association of apocrine glands with hair follicles (APSUs) is maintained, but the association of mammary glands with hair follicles – the presumed ancestral condition – appears to have been lost. In 2002, I hypothesized that this must be due to the inhibition of hair follicle development in the vicinity of mammary glands, and suggested that if the presumptive inhibiting compound (s) could be blocked at the earliest stages of mammary development, hair follicles may develop in association with mammary buds (Oftedal, 2002b). Although the actual signaling pathways are undoubtedly complex, with both shared and differing sensitivities to signaling compounds among different epithelial cell types, it is intriguing that bone morphogenetic proteins (BMPs) inhibit hair follicle formation, and that when Mayer *et al.* (2008) reduced BMP signaling in the mouse by transgenic overexpression of a BMP antagonist, nipple epithelium was converted into pilosebaceous units. Mayer *et al.* (2008) hypothesized that the BMP pathway had been co-opted during evolution of the nipple to suppress hair follicle formation. If so, this occurred following the separation of marsupials and eutherians, given that hair follicle development is not suppressed during nipple development in marsupials; rather, hair follicles subsequently regress and the hairs are shed. Bresslau (1920) even described a developmental stage in the koala, after the nipple has everted (extended above the skin surface), in which a tuft of mammary hairs protrude through galactophores at the apex of the nipple.

Thus, available evidence is consistent with the hypothesis that mammary glands developed from an apocrine-like gland that had an association with hair follicles and sebaceous glands. This is not in conflict with the Vorbach *et al.* (2006) hypothesis that mammary gland secretion had its origin in mucous glands, as the apocrine-like glands themselves must have originated from earlier tetrapod cutaneous glands. The innate immune system itself is of even more ancient origin, with components shared among invertebrates and vertebrates (Beck and Habicht, 1996; Hoffmann *et al.*, 1999; Fujita, 2002), and thus the co-option of innate immune system components into the regulatory elements of epithelial–mesenchyme signaling (McClellan *et al.*, 2008) probably occurred long before the origin of mammary glands.

The evolution of milk constituents

The secretory products of the mammary gland represent the expression of a large number of genes that are upregulated during lactation, but many of these products remain unknown (except as genes) or their functionalities are poorly understood (Smolenski *et al.*, 2007; Lemay *et al.*, 2009). Comparisons of monotreme, marsupial and eutherian genomes suggest that milk and mammary genes tend to be conserved, that is, have not diverged as much as other genes (Lemay *et al.*, 2009). Nonetheless, there are major constituents of milk in these three lineages for which comparative data provide evidence of an evolutionary origin, especially when milk proteins are compared with closely related gene products.

Caseins

All mammalian milks that have been studied contain multiple casein proteins, characterized as α-, β- and κ-caseins. The caseins are phosphorylated during synthesis, and aggregate into large micelles containing calcium bound to phosphorus in calcium phosphate nanoclusters (Smyth *et al.*, 2004). Multiple caseins participate in these micelles; however, κ-casein plays a particularly important role in stabilizing the micelle in secreted milk. The caseins provide a large proportion of the amino acids, calcium, and phosphorus transferred to the young, and via curd formation in the neonatal stomach they play a part in fat and protein digestion. The caseins had already diverged into the three primary types, α-, β-, and κ-caseins, before the separation of monotremes, marsupials and eutherians, as each casein type is found in all three taxa (Rijnkels, 2002 and 2003; Lefevre *et al.*, 2009 and 2010). Thus, caseins have a pre-mammalian origin.

The caseins are members of a much larger family of proteins of unfolded nature that are secreted from cells, usually in association with tissue mineralization or regulation of calcium at target tissues. These proteins, termed secretory calcium-binding phosphoproteins (SCPPs), are secreted by secretory epithelial cells or cells derived from underlying ectomesenchymal cells, and have an ancient history in the evolution of mineralized vertebrate tissues (Kawasaki and Weiss, 2003; Kawasaki, 2009). As unfolded proteins, all SCPPs are low in cysteine and therefore cystine disulfide bridges, and a subclass of the proteins (P/Q-rich SCPPs), including the caseins, are particularly rich in proline and glutamine (Kawasaki and Weiss, 2003; Kawasaki *et al.*, 2011). On the basis of the relative locations and structures of exons of these P/Q-rich SCPPs, as well as their phylogenetic distribution, Kawasaki *et al.* (2011) proposed that the α- and β-caseins derived via gene duplication and exon changes from an ancestral gene (*CSN1/2*) that derives from another *SCPP* gene, either *ODAM* or *SCPPPQ1* (which itself is derived from *ODAM*), whereas κ-casein derives from the *SCPP* gene *FDCSP* (which is also derived from *ODAM*).

Many P/Q-rich SCPPs, including the *ODAM* and *SCPPPQ1* derived proteins, are expressed in mammalian ameloblasts and are involved in mineralization of tooth enamel; follicular dendritic cell secreted peptide (from *FDCSP*) is found in soft connective tissue (periodontal ligament), where it is thought to prevent spontaneous precipitation of calcium phosphate; it is also expressed in the mammary gland (Kawasaki, 2009; Kawasaki *et al.*, 2011). Kawasaki *et al.* (2011) suggest that the initial function of an ancestral SCPP (probably a κ-casein

precursor) in protolacteal secretion may have been to regulate calcium delivery to the surface of an egg and to prevent precipitation of calcium phosphate on the parchment-like eggshell. Kawasaki *et al.* (2011) hypothesize that this may have occurred before the divergence of sauropsids and synapsids, although an ancestral *CSN1/2* has yet to be found in a sauropsid genome.

Subsequently, the types of caseins, and also the numbers of genes involved in producing each type, increased via gene duplication and exon changes (Rijnkels, 2002; Lefevre *et al.*, 2009 and 2010). By an unknown evolutionary process, the different caseins became involved in the formation of complex micelles stabilized by calcium and phosphate bonds (Smyth *et al.*, 2004). This transformation of ancestral SCPPs into a complex of micelle-forming proteins was essential in converting milk from an egg supplement to a major source of nutrients for the suckling young. Given the small size of mammaliaforms in the late Triassic and Jurassic periods, and hence the small size of their eggs (Hopson, 1973; Oftedal, 2002b), the novel nutritive function of these SCPPs must have developed before this time, for example, during the Permian and Triassic periods. This is consistent with the estimated loss of multiple vitellogenin genes beginning approximately 170 mya in the Jurassic period (Brawand *et al.*, 2008). Vitellogenin genes could only be inactivated once the nutrient transport function of the caseins had made egg yolk proteins dispensible.

The Milk fat globule (MFG)

Mammals vary tremendously in the fat content of their milk (from less than 1% in rhinos and some lemurs to 60% in some seals; Oftedal and Iverson, 1995); however, in all species studied, milk lipids are secreted as specialized structures known as MFGs. MFGs are lipid spheres bounded sequentially by a phospholipid monolayer, an inner protein coat, a bilayered phospholipid membrane, and a glycosylated surface (Mather and Keenan, 1998). The collective term for the multi-layered structure or envelope that encloses the lipid sphere is the milk fat globule membrane (MFGM), which is a structure found only in milk. The method of milk lipid secretion appears to be unique, as it has not been found in other organs (McManaman *et al.*, 2006); if this generalization withstands further investigation of lipid secretion in other gland types, the MGM must be considered a key evolutionary novelty of lactation.

What is of particular interest is that the MFGM contains proteins that appear essential to the synthesis and secretion of MFGs. In particular, two proteins, butyrophilin and xanthine oxidoreductase (XOR), play an obligatory structural role in MFGM synthesis, and if they are reduced or eliminated from mouse mammary cells via knockout of the genes that code them, mice fail to produce normal milk; the triacylglycerols within the secretory cells fail to be secreted into milk fat droplets, but rather accumulate in the cytoplasm or leak into the alveolar lumen as unstructured, amorphic lipid masses (Vorbach *et al.*, 2002; Ogg *et al.*, 2004). Although the details of protein–protein interactions during formation of the MFGM are not fully understood (Mather, 2011), these two proteins have apparently been co-opted from other cellular functions during the evolution of the mammary gland.

The butyrophilin in milk is now correctly specified as butyrophilin1A1, as it is the gene product of only one of the genes (*BTN1A*) that code for the family of proteins known as butyrophilins (Rhodes *et al.*, 2001). The butyrophilins are part of the immunoglobulin superfamily and contain two folded immunoglobulin domains, a transmembrane domain and a C-terminal end that may include a large B30.2 domain. In addition to its role in the MFGM, butyrophilin1A1 has been found to be expressed within the thymus; other butyrophilins are more widely expressed among tissues (Smith *et al.*, 2010a). Butyrophilins and related proteins of the immunoglobulin superfamily appear to play a role in the regulation of proliferation, cytokine secretion and activity of T-cells, and butyrophilin1A1 retains this function, at least *in vitro* (Smith *et al.*, 2010a). It is interesting that butyrophilin1A1 is the only butyrophilin that appears to bind XOR via its B30.2 domain, and it is this binding that is believed to be critical to MFG secretion from lactocytes (Jeong *et al.*, 2009). It appears that the ancestral butyrophilin protein was a transmembrane protein in secretory cells that had functions in local immune response, and subsequently evolved a role in synthesis and/or stabilization of the MFGM.

Xanthine oxidoreductase, or XOR, is best known for its role in catalysis of the last two steps in the formation of uric acid, an important nitrogenous waste product, but it has multiple enzymatic functions, and is a member of the molybdoflavoenzyme (MFE) protein family (Garattini *et al.*, 2003). The MFEs are believed to have evolved as an ancestral XOR in prokaryotes (Garattini *et al.*, 2003). Although the XOR gene is sometimes considered to code for a housekeeping protein (Vorbach *et al.*, 2002), this is debatable, as XOR is unequally expressed in cells (Garattini *et al.*, 2003). In mammals, XOR is initially synthesized as the enzyme xanthine dehydrogenase, but it is readily converted to xanthine oxidase, which is the form typically recovered from milk (Enroth *et al.*, 2000; Nishino *et al.*, 2008). Xanthine oxidase generates free radical and reactive nitrogen species, and is upregulated and appears to be during inflammation, leading to the hypotheses that XOR has important antimicrobial activities, perhaps even in milk (Martin *et al.*, 2004), and that XOR may have had an important role in the evolution of innate immunity (Vorbach, 2003). Certainly, XOR and innate immunity are both of pre-eukaryote origin, and were important long before mammary glands evolved. Yet the upregulation and apical membrane localization of XOR in mammary epithelial cells during mammary gland development (McManaman *et al.*, 2002); the binding of XOR to the B30.2 domain of butyrophilin (Jeong *et al.*, 2009); and the failure of MFG formation in heterozygous XOR knockout mice (Vorbach *et al.*, 2002) all indicate a novel function for XOR in the MFGM. Other MFGM components, such as adipophilin, also have important, if incompletely understood, functions (Mather, 2011), and each of these has an evolutionary history.

MFG secretion presumably evolved from some previous form of fat secretion, perhaps by tetrapod or synapsid skin glands.

Certainly, some extant frogs secrete lipids as a means of reducing water loss across the skin (Lillywhite *et al*., 1997; Lillywhite, 2006), and secreted lipids applied to eggs could have had an impact on egg moisture loss (Oftedal, 2002a). However, much more research is required to understand the differences and similarities of secretory mechanisms. Mammary glands bear developmental and structural resemblance to apocrine glands, which led to the hypothesis that mammary glands are derived from ancient apocrine-like glands (Oftedal, 2002b). One can imagine a scenario in which an ancestral apocrine secretion entailed the secretion of apical blebs containing cytoplasm, secretory vesicles and perhaps cytoplasmic lipid droplets, similar to the process described for some specialized apocrine glands, such as human axillary apocrine glands, glands of Moll, ceruminous glands in the outer ear canal, and rodent Harderian glands (Gesase and Satoh, 2003; Stoeckelhuber *et al*., 2003; Stoeckelhuber *et al*., 2006; Stoeckelhuber *et al*., 2011). With an upregulation of mammary fat synthesis, mechanisms may have evolved to minimize cytoplasmic loss to a few cytoplasmic crescents, as in milk secretion. The increase in lipid secretion must have occurred before the miniaturization of the mammaliaforms in the Triassic period and gradually replaced the nutritional role of lipids provided by yolk, allowing the inactivation of vitellogenins involved in transport and storage of lipids in the egg yolk (Brawand *et al*., 2008).

Milk sugar synthesis

All mammalian milks contain at least traces of sugar (Oftedal and Iverson, 1995); in most eutherians the predominant sugar is lactose, whereas in monotremes, marsupials and some eutherian carnivores oligosaccharides predominate (Urashima *et al*., 2001; Messer and Urashima, 2002; Uemura *et al*., 2009; Senda *et al*., 2010). Both lactose and oligosaccharides with lactose at the reducing end are unique to milk (Toba *et al*., 1991) and require a novel synthetic pathway.

In the mammary secretory cell, the synthesis of lactose begins with the synthesis of a unique milk protein, α-lactalbumin, in the rough endoplasmic reticulum (Brew, 2003). α-lactalbumin is then transported to the Golgi apparatus. A transmembrane protein in the trans Golgi, β-1,4-galactosyltransferase1 (β4gal-T1), binds UDP-galactose, producing a conformational change that allows α-lactalbumin to be bound (Ramakrishnan and Qasba, 2001). When α-lactalbumin binds to β4gal-T1, it alters the specificity of β4gal-T1, allowing glucose to become the acceptor sugar for galactose transfer, resulting in the synthesis of lactose. Thus, α-lactalbumin acts as a regulator of β4gal-T1, and without α-lactalbumin β4gal-T1 does not synthesize lactose under physiological conditions (Brew, 2003).

It has been apparent for many years – from amino acid sequence similarity, three-dimensional structure and the structure of the exons that code for α-lactalbumin – that α-lactalbumin is most closely related to c-type lysozyme and is derived from it via gene duplication and base pair substitution (Prager and Wilson, 1988; Qasba and Kumar, 1997; Brew, 2003). The estimated date of origin of α-lactalbumin from c-lysozyme is ancient, before the time of the split of synapsids from sauropsids approximately 310 mya (Prager and Wilson, 1988). Many authors have been puzzled by this date, as it was assumed that mammary glands did not arise until the appearance of 'early mammals' (i.e. mammaliaforms) 100 million or more years later (Hayssen and Blackburn, 1985; Prager and Wilson, 1988; Qasba and Kumar, 1997; Messer and Urashima, 2002), but it is consistent with an ancient origin of lactation.

It is probable that c-lysozyme, as a normal antimicrobial constituent of epithelial secretions and egg white (Callewaert and Michiels, 2010), would have been present in tetrapod and early synapsid skin secretions, including secretions delivered to eggs; a c-type lysozyme is present in amphibian skin secretions (Zhao *et al*., 2006). Although the antimicrobial function of c-lysozyme would presumably help protect eggs (as it does in egg white), what immediate advantage would accrue to eggs or hatchlings from the conversion of c-lysozyme function to that of α-lactalbumin, with the resultant synthesis of lactose? α-lactalbumin does not have lysozyme activity, whereas lysozymes do not bind to β4Gal-T1, because of differences in amino acid composition at key positions involved in binding of substrate (in lysozyme) or binding of β4gal-T1 (in α-lactalbumin; Ramakrishnan and Qasba, 2001; Messer and Urashima, 2002; Brew, 2003; Callewaert and Michiels, 2010), and thus an 'intermediate' with both functions may not have been possible. In addition, one must assume that any lactose thus synthesized would have been indigestible to embryos or hatchlings, given that the intestinal brush-border enzyme lactase could not have evolved without a substrate to digest, and lactose does not occur elsewhere.

Messer and Urashima (2002) argue that the ancestral function of α-lactalbumin as a β4Gal-T1 regulator may have been the production of lactose-containing free oligosaccharides, rather than free lactose *per se*. The amount of α-lactalbumin synthesized in the monotreme mammary gland is minor, and Messer and Urashima (2002) assume this to be the ancestral condition. A wide range of glycosyltransferases would have been present in the trans Golgi of tetrapods and early synapsids, as these are part of the normal synthetic machinery for glycosylation of glycoproteins, glycolipids, and proteoglycans in vertebrates, and they are of ancient origin (Varki, 1998; Lowe and Varki, 1999). A low rate of lactose synthesis, coupled with high activity of other glycosyltransferases that could glycosylate lactose, may have produced free oligosaccharides rather than free lactose, similar to what is observed in extant monotremes and marsupials. Milk oligosaccharides have antimicrobial or probiotic effects, for example by leading pathogens to 'mistake' free oligosaccharides for the oligosaccharide chains of the glycocaylx on apical cell membranes (Newburg, 1996), and thus to fail to bind to these surfaces. Such an effect might benefit the mammary gland, an egg surface, or the digestive tract of a hatchling even before the evolution of the lactase enzymatic mechanism. It is intriguing that marsupial young that consume milk containing oligosaccharides but not lactose do

not have intestinal lactase (Crisp *et al.*, 1989), but whether this is the ancestral mammalian condition is not known.

In eutherians, lactose accumulating in the Golgi apparatus creates an osmotic gradient, which draws water into the Golgi; this aqueous phase (including lactose, α-lactalbumin, other whey proteins, caseins, electrolytes, etc.) is subsequently packaged into secretory vesicles for transport to the apical plasma membrane of the secretory cell (Shennan and Peaker, 2000). This model of milk secretion entails substantial upregulation of α-lactalbumin and lactose synthesis, and the transcription of β4Gal-T1 is also upregulated above constitutively expressed levels via the use of a second transcriptional start site, regulated by a stronger promoter and by more efficient translation of the truncated transcript (Shaper *et al.*, 1998). Although long considered the 'standard' model of milk secretion, this may represent a derived feature of eutherian lactation that only developed after the young evolved the ability to digest lactose. One group of eutherian mammals, the fur seals and sea lions (Pinnipedia: Otariidae), have secondarily lost the ability to synthesize α-lactalbumin because of gene mutations and changes in transcription rates (Sharp *et al.*, 2005; Reich and Arnould, 2007; Sharp *et al.*, 2008); therefore the milk is devoid of lactose or lactose-based oligosaccharides (Oftedal *et al.*, 1987a and 2011). These taxa manage to produce large volumes of high-fat milk (Oftedal *et al.*, 1987b; Arnould and Boyd, 1995; Arnould *et al.*, 1996), but the secretory processes by which the aqueous phase is secreted have not been studied. In mice, knockout of the gene for α-lactalbumin results in a very low level of secretion of high-fat milk, and the offspring do not survive (Stinnakre *et al.*, 1994; Stacey *et al.*, 1995).

Whey proteins as amino acid sources

Caseins have a loosely folded structure with few cystine disulfide bonds, and as a consequence contain a relative deficit of sulfur-containing amino acids (SAA, i.e. methionine and cysteine) relative to the requirements of offspring. In cow's milk, α_s-, β-, and κ-caseins contain approximately 2.9% to 3.7% SAA, by mass, whereas α-lactalbumin and β-lactoglobulin contain approximately 7% to 8% SAA (calculated from data presented in Fox 2003). Suckling mammals appear to require that SAA be present as 4% to 6% of total amino acids in order to attain maximal growth (Foldager *et al.*, 1977; Burns and Milner, 1981; Fuller *et al.*, 1989; National Research Council, 1995). Methionine can substitute for cysteine in most cases (except, perhaps, in premature human infants; Fomon *et al.*, 1986; Thomas *et al.*, 2008), but cysteine can only replace approximately half of the methionine requirement in growing animals (Fuller *et al.*, 1989). In formulating casein-based diets, supplemental cysteine or methionine are required to compensate for the SAA deficit in caseins (e.g. Reeves *et al.*, 1993; National Research Council, 1995). This suggests that other proteins had to coevolve with caseins if milk was to be a balanced source of amino acids, rather than just a supplement. The major milk-specific whey proteins, depending on species, are α-lactalbumin (e.g. in human milk), β-lactoglobulin (e.g. in cow's milk), and WAP (e.g. in rat milk), but additional whey proteins have been identified in marsupials, such as early lactation protein, late lactation protein, and trichosurin (Nicholas *et al.*, 1987; Piotte and Grigor, 1996; Demmer *et al.*, 1998; Piotte *et al.*, 1998).

β-lactoglobulin is the major whey protein in most ruminant milks (including dairy animals such as dairy cattle, goats, sheep and water buffalo), but does not have any indisputable biological role beyond supplying amino acids to the offspring (Sawyer, 2003). As β-lactoglobulin occurs in the milks of monotremes (platypus), several marsupials (brushtail possum, wallabies and kangaroos) and at least 35 species of eutherians, it must have evolved before the divergence of these groups in the Jurassic or Cretaceous period. The discovery that β-lactoglobulin was similar in structure to retinol-binding protein (RBP) led to the hypothesis that β-lactoglobulin might have a role in the transport to the young of vitamin A, vitamin D, fatty acids, or some other essential lipophilic compounds, or may play a role in intestinal uptake of these constituents (Pervaiz and Brew, 1985; Perez and Calvo, 1995; Yang *et al.*, 2009). However, in ruminants, vitamin A is associated with the fat globule and not β-lactoglobulin; in pigs and horses, β-lactoglobulin does not bind either retinol or fatty acids; and, in suckling pups of mice that have not been genetically modified as per Yang *et al.* (2009), vitamin D is obviously absorbed from milk despite the absence of β-lactoglobulin or the pups would develop vitamin D deficiencies. Thus, if β-lactoglobulin has any role in transport and/or intestinal uptake of these lipophilic constituents, it is neither essential nor universal (Perez and Calvo, 1995). A major problem in ascribing a functional role is that β-lactoglobulin is absent from the milks of so many mammals, including laboratory mice and rats, guinea pigs, domestic rabbits, dromedary camels, llamas and humans (Sawyer, 2003).

Both β-lactoglobulin and RBP are members of a large family of small extracellular proteins, termed lipocalins, that have similar tertiary structure, specific amino acid sequence motifs and exon–intron structure of coding genes (Flower, 1996; Akerstrom *et al.*, 2006). This ancient protein family (or superfamily) apparently derives from a bacterial protein and is characterized by a barrel-shaped lipophilic cavern surrounded by a series of eight β-strands, and that is open on one end (Ganfornina *et al.*, 2006). Many lipocalins are known to function via transport and/or sequestration of hydrophobic compounds in this 'barrel' and occasionally at secondary binding sites. Analysis of the molecular evolution of the lipocalins suggests that RBP diverged from the other lipocalins first, followed by β-lactoglobulin, suggesting that β-lactoglobulin is of more ancient origin than other vertebrate lipocalins (other than RBP), including lipocalins that are found in fish and amphibia (Ganfornina *et al.*, 2000; Sanchez *et al.*, 2003 and 2006). It is likely that the ancestral β-lactoglobulin had similar function to that of an ancestral RBP-like protein, that is, transporting hydrophobic compounds in extracellular and/or secreted fluids, long before the appearance of milk as we know it. Although β-lactoglobulin retains a generalized ability to bind a variety of hydrophobic ligands, because of the elasticity of the outer parts of the barrel (Konuma *et al.*, 2007), its current role in

milk appears to be primarily a nutritional one, and in species in which other whey proteins predominate, β-lactoglobulin has become superfluous and has been lost. Two β-lactoglobulin genes have been observed in ruminants, but one is non-coding and is thus a pseudogene (Sawyer, 2003). A β-lactoglobulin pseudogene is also suspected in the human genome, but there may be confusion with the glycodelin gene (Kontopidis *et al.*, 2004). Other lipocalins (trichosurin, late lactation protein) are expressed in marsupial milk (Demmer *et al.*, 1998; Piotte *et al.*, 1998); however, these are only distantly related to β-lactoglobulin and are apparently of more recent origin (Ganfornina *et al.*, 2000).

Among whey proteins, WAP has the highest sulfur amino acid content, approximately 17% to 20% by mass. The key feature of WAP is the presence of two or three domains of approximately 40 to 50 amino acids, each of which contains eight cysteine residues involved in four disulfide bonds; as the domain was first recognized in WAP, it is termed the Whey Acidic Protein Four-Disulphide Core (WFDC) domain. There are at least 33 distinct (non-homologous) proteins among vertebrates and invertebrates that include one to four WFDC domains (see PROSITE, www.expasy.org/cgi-bin/prosite), including proteins with antibacterial, antiviral and anti-inflammatory functions, as well as several proteinase inhibitors. All are secreted proteins, including proteins in respiratory, reproductive and other epithelial secretions (Hagiwara *et al.*, 2003; Bingle *et al.*, 2006). The structural similarity of WAP to other WFDC-containing proteins has led to speculation that WAP may also have antibacterial or proteinase inhibition functions, but attempts to demonstrate this have failed (Hajjoubi *et al.*, 2006; Sharp *et al.*, 2007). There is evidence that WFDC domains influence cell proliferation and growth *in vitro* and in transgenic mice (reviewed by Topcic *et al.*, 2009), but when the WAP gene is deleted in knockout mice, the mice continue to develop normal mammary glands indicating that WAP is not essential for mammary cell differentiation or proliferation (Triplett *et al.*, 2005). The primary effect of WAP deletion in mice appears to be growth retardation of the young during the second half of lactation. Thus, the functional role of WAP in milk, other than as a rich source of sulfur amino acids for the young, remains unclear.

The WFDC domain itself is of ancient origin, being a component in secreted proteins involved in the regulation of shell mineralization in mollusks such as abalone (Treccani *et al.*, 2006), and in antimicrobial response as part of the innate immunity of crustaceans and perhaps insects (Zou *et al.*, 2007; Jia *et al.*, 2008; Smith *et al.*, 2010b). A number of WFDC domain-containing proteins are also secreted by snake venom glands, where they have antibacterial function (Nair *et al.*, 2007; Fry *et al.*, 2008), and by skin glands in frogs, where they serve as antimicrobial defensive compounds (Ali *et al.*, 2002; Zhang *et al.*, 2009). Although much more research is required to determine relationships among invertebrate and vertebrate WFDC-containing proteins, it is likely that an ancestral WAP present in the glandular skin secretion of an egg-tending tetrapod or early synapsid served as a defensive compound against microbes as a component of the innate immune system, similar to existing WFDC proteins in mammalian epididymal, respiratory, and oral mucosal secretions (Hiemstra, 2002; Hagiwara *et al.*, 2003; Bingle and Vyakarnam, 2008), and in frog skin secretions (Ali *et al.*, 2002; Zhang *et al.*, 2009).

As with β-lactoglobulin, it appears that WAP may have lost its purported ancestral function as proteinase inhibitor/antimicrobial protein in skin secretions. Of the two WFDC domains in WAP in eutherian milks, designated as DI and DII, only DII retains the characteristic N-terminal motif found in most WFDC domains (Lys-X-Gly-X-Cys-Pro, where X represents various amino acids); amino acid substitutions in this and other areas of DI may have altered the charge distribution, glycosylation sites, and conformation in such a way that original functions are no longer possible (Ranganathan *et al.*, 1999). Monotreme and marsupial WAPs contain two to three WFDC domains, but of differing sequence and arrangement than eutherian WAPs (Sharp *et al.*, 2007), and it is thought that they may retain functions lost in eutherians, but more evidence is required (Topcic *et al.*, 2009). At least some eutherians have lost WAP in entirety. Although the genes for WAP synthesis have been found in sheep, goats, and cattle, they are missing a nucleotide at the end of the first exon, causing a frameshift mutation (Hajjoubi *et al.*, 2006). They are not transcribed and are thus pseudogenes.

Conclusion

The proposal that mammary secretion has an ancient origin and long evolutionary history (Oftedal, 2002a and 2002b) is increasingly accepted, especially in the face of supportive molecular evidence (Kawasaki and Weiss, 2003; Vorbach *et al.*, 2006; Brawand *et al.*, 2008; McClellan *et al.*, 2008; Capuco and Akers, 2009; Lemay *et al.*, 2009; Lefevre *et al.*, 2010; Kawasaki *et al.*, 2011). It is now possible to formulate a more detailed scenario by which the secreted fluid came to resemble in form and function what we now know as milk. The evolutionary origins of milk appear to be found in the secretions of primitive apocrine-like glands in the skin of early synapsids or even of taxa living before the split of synapsids from sauropsids approximately 310 mya. These glands incorporated elements of the innate immune system in providing protection to the skin and to eggs that were moistened. Membrane and intracellular proteins in secretory epithelial cells, such as butyrophilin and xanthine oxidase, were incorporated into the MFGM, secretory calcium-binding phosphoproteins involved in extracellular regulation of calcium and phosphorus were transformed into casein micelles, and antimicrobial proteins such as lysozyme and WFDC-containing proteins were incorporated as whey proteins, with the transformed lysozyme (i.e. α-lactalbumin) serving to alter sugar synthesis such that new milk-specific sugars were formed. Over a period of perhaps 150 million years, and during the course of multiple radiations of early synapsids and the descendent lineages leading to mammals, this secretory fluid and the glands that produced it became

more complex, the volumes produced became greater, and the extent of dependence by hatchlings and growing young increased. The presence of a glandular skin secretion appears to have been essential to endothermic incubation of eggs with parchment-like shells. The sequential radiations of basal synapsids, therapsids, cynodonts, and mammalialforms became increasingly mammal-like in terms of metabolism, locomotor ability, structural adaptations to diet, and reproduction. On the one hand, the predominance of milk secretion led to reduction in egg yolk mass and smaller and less-developed hatchlings; on the other hand, growth patterns changed as offspring relied for longer periods on milk, with delayed tooth eruption and more specialized mature dentition. It was only because lactation was far advanced that Triassic and Jurassic mammaliaforms could become so tiny, with adult body masses of some taxa weighing only a few grams.

Despite the tremendous diversity in milk composition among extant mammals, the specific constituents of milk are common to the primary mammalian lineages, reflecting that they were inherited from pre-mammalian taxa. The major qualitative differences that have arisen among mammalian taxa (such as lack of α-lactalbumin in fur seals and sea lions, lack of WAP in ruminants, and lack of β-lactoglobulin in humans) are due to losses of functional components, rather than *de novo* creation of new constituents. The evolution of the placenta in eutherians also removed much of the need for nutrient transfer during early development. Marsupials, which have no or very simple placentas, rely on lactation for a greater span of development than eutherians, and not surprisingly, marsupial milks undergo much greater changes in composition over the course of lactation than do most eutherian milks (Oftedal and Iverson, 1995). It is not known whether some of the unique whey proteins that have been found in marsupials represent milk constituents that were lost during eutherian evolution, or if they have evolved since marsupials and eutherians diverged.

When human civilizations finally developed animal agriculture, they were able to select as dairy animals a suite of species (e.g. cows, sheep, goats, water buffaloes, yaks, camels, horses, and asses) characterized by relatively dilute milks containing modest levels of fat, caseins, and whey proteins, and relatively high levels of lactose. Perhaps such species were selected because their milks bore resemblance to human milk, in being dilute, or perhaps such species were easier to milk because their dilute milks accumulated in substantial storage cisterns between bouts of suckling. However, it is only now that we are beginning to appreciate that the remarkable secretory product that is the foundation of the dairy industry had such a very long evolutionary history before mammals appeared on earth.

References

Akerstrom B, Borregaard N, Flower DR and Salier J-P 2006. Lipocalins. Landes Bioscience, Georgetown, TX.

Ali MF, Lips KR, Knoop FC, Fritzsch B, Miller C and Conlon JM 2002. Antimicrobial peptides and protease inhibitors in the skin secretions of the crawfish frog, *Rana areolata*. Biochimica et Biophysica Acta (BBA) – Proteins and Proteomics 1601, 55–63.

Andrechek ER, Mori S, Rempel RE, Chang JT and Nevins JR 2008. Patterns of cell signaling pathway activation that characterize mammary development. Development 135, 2403–2413.

Arnould JPY and Boyd IL 1995. Temporal patterns of milk production in Antarctic fur seals (*Arctocephalus gazella*). Journal of Zoology 237, 1–12.

Arnould JPY, Boyd IL and Socha DG 1996. Milk consumption and growth efficiency in Antarctic fur seal (*Arctocephalus gazella*) pups. Canadian Journal of Zoology 74, 254–266.

Beck G and Habicht GS 1996. Immunity and the invertebrates. Scientific American 275, 60–63, 66.

Beutler B 2004. Innate immunity: an overview. Molecular Immunology 40, 845–859.

Bingle CD and Vyakarnam A 2008. Novel innate immune functions of the whey acidic protein family. Trends in Immunology 29, 444–453.

Bingle L, Cross SS, High AS, Wallace WA, Rassl D, Yuan G, Hellstrom I, Campos MA and Bingle CD 2006. WFDC2 (HE4): a potential role in the innate immunity of the oral cavity and respiratory tract and the development of adenocarcinomas of the lung. Respiratory Research 7, 61–70.

Blackburn DG 2006. Squamate reptiles as model organisms for the evolution of viviparity. Herpetological Monographs 20, 131–146.

Brawand D, Wahli W and Kaessmann H 2008. Loss of egg yolk genes in mammals and the origin of lactation and placentation. PLoS Biology 6, e63. doi:10.1371/journal.pbio.0060063.

Bresslau E 1912. Die entwickelung des mammarapparates der monotremen, marsupialier und einiger placentallier. III. Entwickelung des mammarapparates der marsupialier, insectivoren, nagatheire, carnivoren und widerkauer. Jenaische Denkschriften 7, 647–874, plates 637–646.

Bresslau E 1920. The mammary apparatus of the mammals in light of ontogenesis and phylogenesis. Methuen, London, UK.

Brew K 2003. α-lactalbumin. In Advanced Dairy Chemistry – I. Proteins. Part A (ed. PF Fox and P McSweeney), pp. 387–419. Kluver Academic, New York, NY.

Buckley J, Maunder RJ, Foey A, Pearce J, Val AL and Sloman KA 2010. Biparental mucus feeding: a unique example of parental care in an Amazonian cichlid. The Journal of Experimental Biology 213, 3787–3795.

Burns RA and Milner JA 1981. Sulfur amino acid requirements of immature Beagle dogs. The Journal of Nutrition 111, 2117–2124.

Callewaert L and Michiels CW 2010. Lysozymes in the animal kingdom. Journal of Biosciences 35, 127–160.

Capuco AV and Akers RM 2009. Minireview. The origin and evolution of lactation. Journal of Biology 8, 37. doi:10.1186/jbiol139

Chudinov P 1968. Structure of the integuments of thermomorphs. Doklady Academy of Sciences USSR Earth Science Section 179, 226–229.

Clarke B 1997. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biological Reviews 72, 365–379.

Crisp EA, Messer M and Cowan PE 1989. Intestinal lactase (β-galactosidase) and other disaccharidase activities of suckling and adult common brushtail possums, *Trichosurus vulpecula* (Marsupialia: Phalangeridae). Reproduction, Fertility, and Development 1, 315–324.

Darwin C 1872. On the origin of species by means of natural selection, or the preservation of favoured races in the struggle of life. John Murray, London, UK.

Demmer J, Ross IK, Ginger MR, Piotte CK and Grigor MR 1998. Differential expression of milk protein genes during lactation in the common brushtail possum (*Trichosurus vulpecula*). Journal of Molecular Endocrinology 20, 37–44.

Dhouailly D 2009. A new scenario for the evolutionary origin of hair, feather, and avian scales. Journal of Anatomy 214, 587–606.

Enroth C, Eger BT, Okamoto K, Nishino T and Pai EF 2000. Crystal structures of bovine milk xanthine dehydrogenase and xanthine oxidase: structure-based mechanism of conversion. Proceedings of the National Academy of Sciences of the United States of America 97, 10723–10728.

Flower DR 1996. The lipocalin protein family: structure and function. The Biochemical Journal 318, 1–14.

Foldager J, Huber JT and Bergen WG 1977. Methionine and sulfur amino acid requirement in the preruminant calf. Journal of Dairy Science 60, 1095–1104.

Fomon SJ, Ziegler EE, Nelson SE and Edwards BB 1986. Requirement for sulfur-containing amino acids in infancy. The Journal of Nutrition 116, 1405–1422.

Fox PF 2003. Milk proteins: general and historical aspects. In Advanced dairy chemistry – I. Proteins. Part A (ed. PF Fox and P McSweeney), pp. 1–48. Kluwer Academic, New York, NY.

Fry BG, Scheib H, van der Weerd L, Young B, McNaughtan J, Ramjan SFR, Vidal N, Poelmann RE and Norman JA 2008. Evolution of an arsenal: structural and functional diversification of the venom system in the advanced snakes (Caenophidia). Molecular & Cellular Proteomics 7, 215–246.

Fujita T 2002. Evolution of the lectin–complement pathway and its role in innate immunity. Nature Reviews Immunology 2, 346–353.

Fuller MF, McWilliam R, Wang TC and Giles LR 1989. The optimum dietary amino acid pattern for growing pigs. 2. Requirements for maintenance and for tissue protein accretion. The British Journal of Nutrition 62, 255–267.

Ganfornina MD, Gutierrez G, Bastiani M and Sanchez D 2000. A phylogenetic analysis of the lipocalin protein family. Molecular Biology and Evolution 17, 114–126.

Ganfornina MD, Sanchez D, Greene LH and Flower DR 2006. The Lipocalin protein family: protein sequence, structure and relationship to the calycin superfamily. In Lipocalins (ed. B Akerstrom, N Borregaard, DR Flower and J-P Salier), pp. 17–27. Landes Bioscience, Georgetown, TX.

Garattini E, Mendel R, RomÃ£o MJ, Wright R and Terao M 2003. Mammalian molybdo-flavoenzymes, an expanding family of proteins: structure, genetics, regulation, function and pathophysiology. Biochemical Journal 372, 15–32.

Gesase AP and Satoh Y 2003. Apocrine secretory mechanism: recent findings and unresolved problems. Histology and Histopathology 18, 597–608.

Gregory WK 1910. The orders of mammals. Bulletin of the American Museum of Natural History 27, 1–524.

Griffiths M 1978. Biology of the monotremes. Academic Press, New York, NY.

Gritli-Linde A, Hallberg K, Harfe BD, Reyahi A, Kannius-Janson M, Nilsson J, Cobourne MT, Sharpe PT, McMahon AP and Linde A 2007. Abnormal hair development and apparent follicular transformation to mammary gland in the absence of hedgehog signaling. Developmental Cell 12, 99–112.

Hagiwara K, Kikuchi T, Endo Y, Huqun, Usui K, Takahashi M, Shibata N, Kusakabe T, Xin H, Hoshi S, Miki M, Inooka N, Tokue Y and Nukiwa T 2003. Mouse SWAM1 and SWAM2 are antibacterial proteins composed of a single whey acidic protein motif. Journal of Immunology 170, 1973–1979.

Hajjoubi S, Rival-Gervier S, Hayes H, Floriot S, Eggen A, Piumi F, Chardon P, Houdebine LM and Thepot D 2006. Ruminants genome no longer contains whey acidic protein gene but only a pseudogene. Gene 370, 104–112.

Hatsell SJ and Cowin P 2006. Gli3-mediated repression of Hedgehog targets is required for normal mammary development. Development 133, 3661–3670.

Hayssen V and Blackburn DG 1985. α-lactalbumin and the origins of lactation. Evolution 39, 1147–1149.

Hiemstra PS 2002. Novel roles of protease inhibitors in infection and inflammation. Biochemical Society Transactions 30, 116–120.

Hoffmann JA, Kafatos FC, Janeway CA and Ezekowitz RA 1999. Phylogenetic perspectives in innate immunity. Science 284, 1313–1318.

Hood WR, Oftedal OT and Kunz TH 2011. Is tissue maturation necessary for flight? Changes in body composition during postnatal development in the big brown bat. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 181, 423–435.

Hopson JA 1973. Endothermy, small size and the origin of mammalian reproduction. American Naturalist 107, 446–452.

Horseman ND and Buntin JD 1995. Regulation of pigeon cropmilk secretion and parental behaviors by prolactin. Annual Review of Nutrition 15, 213–238.

Jenssen H, Hamill P and Hancock REW 2006. Peptide antimicrobial agents. Clinical Microbiology Reviews 19, 491–511.

Jeong J, Rao AU, Xu J, Ogg SL, Hathout Y, Fenselau C and Mather IH 2009. The PRY/SPRY/B30. 2 Domain of butyrophilin 1A1 (BTN1A1) binds to xanthine oxidoreductase. Journal of Biological Chemistry 284, 22444–22456.

Jia Y, Sun Y, Wang Z, Wang Q, Wang X, Zhao X and Wang J 2008. A single whey acidic protein domain (SWD)-containing peptide from fleshy prawn with antimicrobial and proteinase inhibitory activities. Aquaculture 284, 246–259.

Kawasaki K 2009. The SCPP gene repertoire in bony vertebrates and graded differences in mineralized tissues. Development Genes and Evolution 219, 147–157.

Kawasaki K and Weiss KM 2003. Mineralized tissue and vertebrate evolution: the secretory calcium-binding phosphoprotein gene cluster. Proceedings of the National Academy of Sciences of the United States of America 100, 4060–4065.

Kawasaki K, Lafont A and Sire J 2011. The evolution of milk casein genes from tooth genes before the origin of mammals. Molecular Biology and Evolution 28, 2053–2061.

Kemp TS 2005. The origin and evolution of mammals. Oxford University Press, New York, NY.

Kontopidis G, Holt C and Sawyer L 2004. Invited review: beta-lactoglobulin: binding properties, structure, and function. Journal of Dairy Science 87, 785–796.

Konuma T, Sakurai K and Goto Y 2007. Promiscuous binding of ligands by β-lactoglobulin involves hydrophobic interactions and plasticity. Journal of Molecular Biology 368, 209–218.

Kupfer A, Müller H, Antoniazzi MM, Jared C, Greven H, Nussbaum RA and Wilkinson M 2006. Parental investment by skin feeding in a caecilian amphibian. Nature 440, 926–929.

Lefevre CM, Sharp JA and Nicholas KR 2009. Characterisation of monotreme caseins reveals lineage-specific expansion of an ancestral casein locus in mammals. Reproduction, Fertility, and Development 21, 1015–1027.

Lefevre CM, Sharp JA and Nicholas KR 2010. Evolution of lactation: ancient origin and extreme adaptations of the lactation system. Annual Review of Genomics and Human Genetics 11, 219–238.

Lemay DG, Lynn DJ, Martin WF, Neville MC, Casey TM, Rincon G, Kriventseva EV, Barris WC, Hinrichs AS, Molenaar AJ, Pollard KS, Maqbool NJ, Singh K, Murney R, Zdobnov EM, Tellam RL, Medrano JF, German JB and Rijnkels M 2009. The bovine lactation genome: insights into the evolution of mammalian milk. Genome Biology 10, R43. doi:10.1186/gb-2009-10-4-r43.

Lillywhite HB 2006. Water relations of tetrapod integument. The Journal of Experimental Biology 209, 202–226.

Lillywhite HB, Mittal AK, Garg TK and Agrawal N 1997. Integumentary structure and its relationship to wiping behaviour in the common Indian tree frog, *Polypedates maculatus*. Journal of Zoology 243, 675–687.

Lourdais O, Hoffman TCM and Denardo DF 2007. Maternal brooding in the children's python (*Antaresia childreni*) promotes egg water balance. Journal of Comparative physiology B: Biochemical, Systemic, and Environmental Physiology 177, 569–577.

Lowe JB and Varki A 1999. Glycosyltransferases. In Essentials of glycobiology (ed. A Varki, R Cummings, J Esko, H Freeze, G Hart and J Marth), pp. 253–266. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Luo ZX, Crompton AW and Sun AL 2001. A new mammaliaform from the early Jurassic and evolution of mammalian characteristics. Science 292, 1535–1540.

Martin HM, Hancock JT, Salisbury V and Harrison R 2004. Role of xanthine oxidoreductase as an antimicrobial agent. Infection and Immunity 72, 4933–4939.

Mather IH 2011. Milk lipids. Milk fat globule membrane. In Encyclopedia of dairy sciences (ed. J Fuquay, PF Fox and P McSweeney), vol. 3, 2nd edition, pp. 680–690. Academic Press, San Diego.

Mather IH and Keenan TW 1998. Origin and secretion of milk lipids. Journal of Mammary Gland Biology and Neoplasia 3, 259–273.

Mayer JA, Foley J, De La Cruz D, Chuong C-M and Widelitz R 2008. Conversion of the nipple to hair-bearing epithelia by lowering bone morphogenetic protein pathway activity at the dermal–epidermal interface. The American Journal of Pathology 173, 1339–1348.

McClellan HL, Miller SJ and Hartmann PE 2008. Evolution of lactation: nutrition v. protection with special reference to five mammalian species. Nutrition Research Reviews 21, 97–116.

McManaman JL, Reyland ME and Thrower EC 2006. Secretion and fluid transport mechanisms in the mammary gland: comparisons with the exocrine pancreas and the salivary gland. Journal of Mammary Gland Biology and Neoplasia 11, 249–268.

McManaman JL, Palmer CA, Wright RM and Neville MC 2002. Functional regulation of xanthine oxidoreductase expression and localization in the mouse mammary gland: evidence of a role in lipid secretion. The Journal of Physiology 545, 567–579.

Messer M and Urashima T 2002. Evolution of milk oligosaccharides and lactose. Trends in Glycoscience and Glycotechnology 14, 153–176.

Nair DG, Fry BG, Alewood P, Kumar PP and Kini RM 2007. Antimicrobial activity of omwaprin, a new member of the waprin family of snake venom proteins. The Biochemical Journal 402, 93–104.

National Research Council 1995. Nutrient requirements of laboratory animals. National Academy Press, Washington, DC.

Nelson CM and Bissell MJ 2006. Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. Annual Review of Cell and Developmental Biology 22, 287–309.

Newburg DS 1996. Oligosaccharides and glycoconjugates in human milk: their role in host defense. Journal of Mammary Gland Biology and Neoplasia 1, 271–283.

Nicholas KR, Messer M, Elliott C, Maher F and Shaw DC 1987. A novel whey protein synthesized only in late lactation by the mammary gland from the tammar (*Macropus eugenii*). The Biochemical Journal 241, 899–904.

Nishino T, Okamoto K, Eger BT, Pai EF and Nishino T 2008. Mammalian xanthine oxidoreductase – mechanism of transition from xanthine dehydrogenase to xanthine oxidase. The FEBS Journal 275, 3278–3289.

Novacek MJ, Rougier GW, Wible JR, McKenna MC, Dashzeveg D and Horovitz I 1997. Epipubic bones in eutherian mammals from the Late Cretaceous of Mongolia. Nature 389, 483–486.

Oftedal OT 1997. Lactation in whales and dolphins: evidence of divergence between baleen- and toothed-species. Journal of Mammary Gland Biology and Neoplasia 2, 205–230.

Oftedal OT 2000. Use of maternal reserves as a lactation strategy in large mammals. Proceedings of the Nutrition Society 59, 99–106.

Oftedal OT 2002a. The origin of lactation as a water source for parchment-shelled eggs. Journal of Mammary Gland Biology and Neoplasia 7, 253–266.

Oftedal OT 2002b. The mammary gland and its origin during synapsid evolution. Journal of Mammary Gland Biology and Neoplasia 7, 225–252.

Oftedal OT 2011. Milk of marine mammals. In Encyclopedia of dairy sciences (ed. J Fuquay, PF Fox and P McSweeney), vol. 3, 2nd edition, pp. 563–580. Academic Press, San Diego, CA.

Oftedal OT and Gittleman JL 1989. Patterns of energy output during reproduction in carnivores. In Carnivore behavior, ecology and evolution (ed. JL Gittleman), pp. 355–378. Cornell University Press, Ithaca, NY.

Oftedal OT and Iverson SJ 1995. Comparative analysis of non-human milks. A. phylogenetic variation in the gross composition of milks. In Handbook of milk composition (ed. RG Jensen), pp. 749–789. Academic Press, San Diego, CA.

Oftedal OT, Boness DJ and Tedman RA 1987a. The behavior, physiology, and anatomy of lactation in the Pinnipedia. Current Mammalogy 1, 175–245.

Oftedal OT, Iverson SJ and Boness DJ 1987b. Milk and energy intakes of suckling California sea lion *Zalophus californianus* pups in relation to sex, growth, and predicted maintenance requirements. Physiological Zoology 60, 560–575.

Oftedal OT, Bowen DW and Boness DJ 1993a. Energy transfer by lactating hooded seals and nutrient deposition in their pups during the 4 days from birth to weaning. Physiological Zoology 66, 412–436.

Oftedal OT, Alt GL, Widdowson EM and Jakubasz MR 1993b. Nutrition and growth of suckling black bears (*Ursus americanus*) during their mothers' winter fast. British Journal of Nutrition 70, 59–79.

Ogg SL, Weldon AK, Dobbie L, Smith AJH and Mather IH 2004. Expression of butyrophilin (Btn1a1) in lactating mammary gland is essential for the regulated secretion of milk-lipid droplets. Proceedings of the National Academy of Sciences of the United States of America 101, 10084–10089.

Packard MJ and Seymour RS 1997. Evolution of the amniote egg. In Amniote origins: completing the transition to land (ed. SS Sumida and KLM Martin), pp. 265–290. Academic Press, San Diego, CA.

Perez MD and Calvo M 1995. Interaction of beta-lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: a review. Journal of Dairy Science 78, 978–988.

Pervaiz S and Brew K 1985. Homology of β-lactoglobulin, serum retinol-binding protein, and protein HC. Science 228, 335–337.

Piotte CP and Grigor MR 1996. A novel marsupial protein expressed by the mammary gland only during the early lactation and related to the Kunitz proteinase inhibitors. Archives of Biochemistry and Biophysics 330, 59–64.

Piotte CP, Hunter AK, Marshall CJ and Grigor MR 1998. Phylogenetic analysis of three lipocalin-like proteins present in the milk of *Trichosurus vulpecula* (Phalangeridae, Marsupialia). Journal of Molecular Evolution 46, 361–369.

Pond CM 1977. The significance of lactation in the evolution of mammals. Evolution 31, 177–199.

Prager EM and Wilson AC 1988. Ancient origin of lactalbumin from lysozyme: analysis of DNA and amino acid sequences. Journal of Molecular Evolution 27, 326–335.

Qasba PK and Kumar S 1997. Molecular divergence of lysozymes and α-lactalbumin. Critical Reviews in Biochemistry and Molecular Biology 32, 255–306.

Quagliata S, Malentacchi C, Delfino C, Brunasso AMG and Delfino G 2006. Adaptive evolution of secretory cell lines in vertebrate skin. Caryologia 59, 187–206.

Ramakrishnan B and Qasba PK 2001. Crystal structure of lactose synthase reveals a large conformational change in its catalytic component, the β-1,4-galactosyltransferase-I. Journal of Molecular Biology 310, 205–218.

Ranganathan S, Simpson KJ, Shaw DC and Nicholas KR 1999. The whey acidic protein family: a new signature motif and three-dimensional structure by comparative modeling. Journal of Molecular Graphics & Modelling 17, 106–113, 134–136.

Reeves PG, Nielsen FH and Fahey GC 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. The Journal of Nutrition 123, 1939–1951.

Reich C and Arnould J 2007. Evolution of Pinnipedia lactation strategies: a potential role for α-lactalbumin? Biology Letters 3, 546–549.

Reilly SM and White TD 2003. Hypaxial motor patterns and the function of epipubic bones in primitive mammals. Science 299, 400–402.

Rhodes DA, Stammers M, Malcherek G, Beck S and Trowsdale J 2001. The cluster of BTN genes in the extended major histocompatibility complex. Genomics 71, 351–362.

Rijnkels M 2002. Multispecies comparison of the casein gene loci and evolution of casein gene family. Journal of Mammary Gland Biology and Neoplasia 7, 327–345.

Rijnkels M 2003. Multispecies comparative analysis of a mammalian-specific genomic domain encoding secretory proteins. Genomics 82, 417–432.

Robinson GW 2004. Identification of signaling pathways in early mammary gland development by mouse genetics. Breast Cancer Research 6, 105–108.

Sanchez D, Ganfornina MD, Gutierrez G and Marin A 2003. Exon–intron structure and evolution of the Lipocalin gene family. Molecular Biology and Evolution 20, 775–783.

Sanchez D, Ganfornina MD, Gutierrez G, Gauthier-Jauneau A-C, Risler J-L and Salier J-P 2006. Lipocalin genes and their evolutionary history. In Lipocalins (ed. B Akerstrom, N Borregaard, DR Flower and J-P Salier), pp. 5–16. Landes Bioscience, Georgetown, TX.

Sawyer L 2003. β-lactoglobulin. In Advanced dairy chemistry – I. Proteins. Part A (ed. PF Fox and P McSweeney), pp. 319–386. Kluwer Academic, New York, NY.

Senda A, Hatakeyama E, Kobayashi R, Fukuda K, Uemura Y, Saito T, Packer C, Oftedal OT and Urashima T 2010. Chemical characterization of milk oligosaccharides of an African lion (*Panthera leo*) and a clouded leopard (*Neofelis nebulosa*). Animal Science Journal 81, 687–693.

Shaper NL, Charron M, Lo NW and Shaper JH 1998. β-1,4-galactosyltransferase and lactose biosynthesis: recruitment of a housekeeping gene from the nonmammalian vertebrate gene pool for a mammary gland specific function. Journal of Mammary Gland Biology and Neoplasia 3, 315–324.

Sharp JA, Lefevre C and Nicholas KR 2007. Molecular evolution of monotreme and marsupial whey acidic protein genes. Evolution & Development 9, 378–392.

Sharp JA, Lefevre C and Nicholas KR 2008. Lack of functional alpha-lactalbumin prevents involution in Cape fur seals and identifies the protein as an apoptotic milk factor in mammary gland involution. BMC Biology 6, 48. doi:10.1186/1741-7007-6-48.

Sharp JA, Cane KN, Lefevre C, Arnould JPY and Nicholas KR 2005. Fur seal adaptations to lactation: insights into mammary gland function. Current Topics in Developmental Biology 72, 275–308.

Shennan DB and Peaker M 2000. Transport of milk constituents by the mammary gland. Physiological Reviews 80, 925–951.

Sidor CA and Hopson JA 1998. Ghost lineages and "mammalness": assessing the temporal pattern of character acquisition in the Synapsida. Paleobiology 24, 254–273.

Smith IA, Knezevic BR, Ammann JU, Rhodes DA, Aw D, Palmer DB, Mather IH and Trowsdale J 2010a. BTN1A1, the mammary gland butyrophilin, and BTN2A2 are both inhibitors of T cell activation. The Journal of Immunology 184, 3514–3525.

Smith VJ, Desbois AP and Dyrynda EA 2010b. Conventional and unconventional antimicrobials from fish, marine invertebrates and micro-algae. Marine Drugs 8, 1213–1262.

Smolenski G, Haines S, Fiona YSK, Bond J, Farr V, Davis SR, Stelwagen K and Wheeler TT 2007. Characterisation of host defence proteins in milk using a proteomic approach. Journal of Proteome Research 6, 207–215.

Smyth E, Clegg RA and Holt C 2004. A biological perspective on the structure and function of caseins and casein micelles. International Journal of Dairy Technology 57, 121–126.

Stacey A, Schnieke A, Kerr M, Scott A, McKee C, Cottingham I, Binas B, Wilde C and Colman A 1995. Lactation is disrupted by α-lactalbumin deficiency and can be restored by human α-lactalbumin gene replacement in mice. Proceedings of the National Academy of Sciences of the United States of America 92, 2835–2839.

Starck JM and Ricklefs R 1998. Avian growth and development: evolution within the altricial-precocial spectrum. Oxford University Press, New York, NY.

Stinnakre MG, Vilotte JL, Soulier S and Mercier JC 1994. Creation and phenotypic analysis of alpha-lactalbumin-deficient mice. Proceedings of the National Academy of Sciences of the United States of America 91, 6544–6548.

Stoeckelhuber M, Stoeckelhuber BM and Welsch U 2003. Human glands of Moll: histochemical and ultrastructural characterization of the glands of Moll in the human eyelid. Journal of Investigative Dermatology 121, 28–36.

Stoeckelhuber M, Schubert C, Kesting MR, Loeffelbein DJ, Nieberler M, Koehler C and Welsch U 2011. Human axillary apocrine glands: proteins involved in the apocrine secretory mechanism. Histology and Histopathology 26, 177–184.

Stoeckelhuber M, Matthias C, Andratschke M, Stoeckelhuber BM, Koehler C, Herzmann S, Sulz A and Welsch U 2006. Human ceruminous gland: ultrastructure and histochemical analysis of antimicrobial and cytoskeletal components. The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology 288A, 877–884.

Taigen TL, Pough FH and Stewart MM 1984. Water balance of terrestrial anuran (*Eleutherodactylus coqui*) eggs: importance of parental care. Ecology 65, 248–255.

Thomas B, Gruca LL, Bennett C and Parimi PS 2008. Metabolism of methionine in the newborn infant: response to the parenteral and enteral administration of nutrients. Pediatric Research 64, 381–386.

Thompson MB, Biazik JB, Lui S, Adams SM and Murphy CR 2006. Morphological and functional changes to the uterus of lizards with different placental complexities. Herpetological Monographs 20, 178–185.

Toba T, Nagashima S and Adachi S 1991. Is lactose really present in plants? Journal of the Science of Food and Agriculture 54, 305–308.

Topcic D, Auguste A, De Leo AA, Lefevre C, Digby MR and Nicholas KR 2009. Characterization of the tammar wallaby (*Macropus eugenii*) whey acidic protein gene; new insights into the function of the protein. Evolution & Development 11, 363–375.

Treccani L, Mann K, Heinemann F and Fritz M 2006. Perlwapin, an abalone nacre protein with three four-disulfide core (whey acidic protein) domains, inhibits the growth of calcium carbonate crystals. Biophysical Journal 91, 2601–2608.

Triplett AA, Sakamoto K, Matulka LA, Shen L, Smith GH and Wagner KU 2005. Expression of the whey acidic protein (Wap) is necessary for adequate nourishment of the offspring but not functional differentiation of mammary epithelial cells. Genesis 43, 1–11.

Tyndale-Biscoe H and Renfree M 1987. Reproductive physiology of marsupials. Cambridge University Press, Cambridge, UK.

Uemura Y, Takahashi S, Senda A, Fukuda K, Saito T, Oftedal OT and Urashima T 2009. Chemical characterization of milk oligosaccharides of a spotted hyena (*Crocuta crocuta*). Comparative Biochemistry and Physiology A: Molecular & Integrative Physiology 152, 158–161.

Urashima T, Saito T, Nakamura T and Messer M 2001. Oligosaccharides of milk and colostrum in non-human mammals. Glycoconjugate Journal 18, 357–371.

Varki A 1998. Factors controlling the glycosylation potential of the Golgi apparatus. Trends in Cell Biology 8, 34–40.

Vorbach C 2003. Xanthine oxidoreductase is central to the evolution and function of the innate immune system. Trends in Immunology 24, 512–517.

Vorbach C, Scriven A and Capecchi MR 2002. The housekeeping gene xanthine oxidoreductase is necessary for milk fat droplet enveloping and secretion: gene sharing in the lactating mammary gland. Genes & Development 16, 3223–3235.

Vorbach C, Capecchi MR and Penninger JM 2006. Evolution of the mammary gland from the innate immune system? BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology 28, 606–616.

Watson CJ and Khaled WT 2008. Mammary development in the embryo and adult: a journey of morphogenesis and commitment. Development 135, 995–1003.

West KL, Oftedal OT, Carpenter JR, Krames BJ, Campbell M and Sweeney JC 2007. Effect of lactation stage and concurrent pregnancy on milk composition in the bottlenose dolphin. Journal of Zoology 273, 148–160.

Yang MC, Chen NC, Chen C-J, Wu CY and Mao SJT 2009. Evidence for beta-lactoglobulin involvement in vitamin D transport in vivo – role of the gamma-turn (Leu-Pro-Met) of beta-lactoglobulin in vitamin D binding. FEBS Journal 276, 2251–2265.

Zhang Z, Zhang B, Nie X, Liu Q, Xie F and Shang D 2009. Transcriptome analysis and identification of genes related to immune function in skin of the Chinese brown frog. Zoological Science 26, 80–86.

Zhao Y, Jin Y, Lee W and Zhang Y 2006. Purification of a lysozyme from skin secretions of *Bufo andrewsi*. Comparative Biochemistry and Physiology C: Toxicology & Pharmacology 142, 46–52.

Zou Z, Evans JD, Lu Z, Zhao P, Williams M, Sumathipala N, Hetru C, Hultmark D and Jiang H 2007. Comparative genomic analysis of the *Tribolium* immune system. Genome Biology 8, R177. doi:10.1186/gb-2007-8-8-r177.