The evolution of the mammary gland

by

Jan FREUND

Promotors: Xanthippe Boulougouris
Prof. dr. Christian Burvenich

Literature Review as part of the Master's Dissertation

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At first, I want to thank Hanna Bellmann and our daughter Veerle for their support and inspiration during the process of writing this thesis. I would also like to thank Petra Bellmann, Rebekka Bellmann, Andreas Roth and Felicia Benefield for their interest in this thesis, their suggestions and corrections. Further, I want to give thanks to my promotors, Xanthippe Boulougouris and Prof. dr, Christian Burvenich, for their support.
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SUMMARY

All modern mammals have mammary glands in order to nurture their young with milk. Milk has protective properties and is important for immunological maturation. Lactation and its various functions did not come about through a saltational de novo evolution, but as the result of a gradual evolutionary process. The origins of this process are difficult to follow up because of the lack of fossil evidence. 200 million years ago however, a complex lactation system had already been established. Thus, the mammary gland evolved along the synapsid lineage after the split between synapsids and sauropsids and before the last common ancestor of today's mammals. Hypotheses on its original function remain speculative but its nutritive function probably evolved subsequently to another function. Proposed and often cited scenarios are the evolution from a skin gland that produced either an antimicrobial secretion or a watery secretion in order to moisturize eggs. The mammary gland is often compared with apocrine glands because of their similar mechanism of secretion but it is also associated (in monotremes, embryological in others mammals) with sebaceous glands and hair. Therefore, it might be derived from an apo-pilo-sebaceous unit, an ancestral structure to both apocrine and sebaceous glands. Recently, molecular and genetic research has unraveled the evolutionary history of some important milk constituents, showing that the evolution of caseins and α-lactalbumin began 400-300 million years ago.

Key words: Evolution – Lactation – Mammals – Mammary Gland – Milk

SAMENVATTING

De melkklier is het meest karakteristieke kenmerk van alle zoogdieren. Ze voeden hun jong met melk, welke bovendien een belangrijke beschermende en immunologische functie vervult. De drie vandaag voorkomende claden van zoogdieren, Monotremata (cloacadieren), Marsupialia (buideldieren) en Eutheria (placentadieren), hebben allemaal een functionerende melkklier. Niet te min zijn er morfologische en functionele verschillen. Zo hebben de eierleggende monotremen geen tepel en zijn hun melkklieren geassocieerd met haar. Deze kenmerken werden als voorouderlijk beschouwd en waren al aanwezig in de laatste gemeenschappelijke voorouder van de huidige Mammalia 220 miljoen jaar geleden. Ongeveer 200 miljoen jaar geleden bestond er dus al een complexe melkklier. De stamboom van de zoogdieren gaat terug op de Synapsida die 310 miljoen jaar geleden splitsten van de Sauropsida (voorouders van de huidige schubreptielen, krokodilachtigen, schildpadden en vogels). Ergens in deze periode van 310 miljoen jaar geleden tot 200 miljoen jaar geleden heeft de evolutie van de melkklier plaatsgevonden. Dit was geen plotselinge gebeurtenis maar een gradueel proces waarin op al bestaande structuren opgebouwd werd. Omdat er geen fossielen van weke delen zoals de melkklier bestaan, zijn de meeste hypotheses over het ontstaan van de melkklier speculatief. Maar er zijn aanwijzingen dat Synapsida een glandulair integument erfden van hun Tetrapoda voorouders. De synapsida waren dus pre-geadapteerd om lactatie te ontwikkelen. Er bestaan verschillende hypotheses over de oorspronkelijke functie van de primitieve melk en over het weefsel waarvan de oorspronkelijke melkklier zou afkomstig zijn. Het is niet mogelijk om met zekerheid te zeggen wat de
ware eerste functie was. Maar het lijkt waarschijnlijk dat synapsiden hun perkamentachtige eieren met een waterig secret voorzagen dat de eieren hetzij tegen uitdroging, hetzij tegen microbiële pathogenen beschermde. Deze twee hypothesen lijken het meest plausibel omdat dat eieren met een perkamentachtig schaal gevoelig zijn aan uitdroging en omdat melk van de huidige zoogdieren ook een groot aantal antimicrobiële moleculen bevat. Sommige van deze moleculen, zoals α-lactalbumin en lysozyme, hebben een lange evolutionaire geschiedenis en waren al aanwezig in vroege synapsiden. De nutritionele functie van melk evolueerde waarschijnlijk pas secundair. Verder zouden feromonen en rol hebben gespeeld in het ontstaan van het zogen. Over de weefselorigine bestaan er eveneens verschillende hypothesen. Omdat de evolutie van lactatie waarschijnlijk door coöpération van een reeds bestaande secretie mechanisme gebeurd heeft men de melkklier met andere huidklieren vergeleken. De melkklier beschikt bijvoorbeeld zoals apocriene zweetklieren over twee cellagen (één laag secretorisch epitheel omgeven door myoepitheliaal cellen) ter hoogte van het kliergedeelte en kan haar producten op apocriene en merocriene wijze afscheiden. Eerst komen apocriene klieren vaak in een triade van apocriene klier, haarfollikel en talgklier voor (apo-pilo-sebaceous eenheid). Deze associatie bestaat ook bij de melkklier (mammo-pilo-sebaceous eenheid). Dit werd eerst aangetoond bij monotremen, maar ondertussen is dit ook tijdens de embryologische ontwikkeling van buideldieren en placentadieren aangetoond. Bij de laatste twee verdwijnt de associatie met haar evenwel tijdens de ontwikkeling. Waarschijnlijk zal de melkklier dus afkomstig zijn van een voorganger van zowel de apocriene klier als ook de melkklier. Recent werden de melk- en melkkliergenen van verschillende zoogdieren vergeleken, waarbij opviel dat deze genen geconserveerd zijn. Er is weinig variatie in deze genen wat erop wijst dat ze essentieel zijn en dat de evolutie van de melkklier beperkt wordt om de overlevingskans van moeder en nakomelingen te maximaliseren.

Key words: Evolution – Lactatie – Melk – Melkklier – Zoogdieren
INTRODUCTION

The mammary gland is the most prominent characteristic unifying all of today's mammals. Thus, it is hardly surprising that Carl von Linné named the class of animals that "suckle their young by means of lactiferous teats" (Linné 1806) Mammalia. In 1872, Charles Darwin devoted a chapter of his sixth edition of The Origin of Species to the rebuttal of objections to his theory of natural selection. In it, he gives one of the first explanations of how a mammary gland could have evolved by replying to the following objection by Mivart, who was one of Darwin’s main critics at the time.

Mr. Mivart asks; "Is it conceivable that the young of any animal was ever saved from destruction by accidentally sucking a drop scarcely nutritious fluid from an accidentally hypertrophied cutaneous gland of its mother? And even if one was so, what chance was there for the perpetuation of such a variation?"

Mivart’s question shows how difficult it was and maybe still is to imagine a process that gave rise to such a complex and essential organ as the mammary gland. Darwin's response, knowing well that at the time nothing certain could be said about mammary gland evolution, was that the mammary gland might have evolved within the marsupial sack of a possible marsupial ancestor (Darwin 1872). There nutritious secretions by cutaneous glands might have provided a selective advantage. Over the course of time, these glands might have clustered in a process of specialization to form mammary glands that originally had no nipples as seen in the platypus (Darwin 1872). Today Darwin’s explanation for the evolution of a mammary gland is outdated, as more is known about mammalian evolution and the origin of the mammary gland, but his theory of evolution remains one of the pillars of modern biology.

With a more conclusive fossil record much more is known about evolution and the mammalian ancestry. Nevertheless, there are still questions unanswered about the evolution of the mammary gland, as there are no sufficient fossils of soft tissues. Therefore, even more recent hypotheses on the origin of the mammary gland remain speculative and have little predictive value, but instead give plausible explanations about the evolution of the mammary gland (Blackburn et al. 1989).

Phylogenetic approaches allow pinpointing when different clades split and we can make approximations as to when a certain trait had been established (Madsen 2009; Lefèvre et al. 2010). Recently, milk protein and mammary genes were compared across different species and clades unveiling the genetic ancestry of some milk proteins and lactation (Lemay et al. 2009; Kawasaki et al. 2011).

In this review I will integrate what is known today about mammary gland evolution and what might help to answer the following questions.

1. "When did a mammary gland first evolve, and from which original structure?"
2. "What was its original function?"
3. "How did it evolve in the course of time?"

In the discussion, I go into more detail on how the genetic and molecular methods might help to unravel the evolution of the mammary gland.
LITERATURE REVIEW

1. BACKGROUND

1.1. THE DESCENT OF MAMMALS

The term Mammalia is today often reserved for the three living clades of mammals and their last common ancestor (Benton 2005). These three living clades are the Proterotheria (monotremes), the Metatheria (marsupials) and the Eutheria (placentals) (Bledges, 2009). These groups are the only existing descendants of the synapsids, which first appeared approximately 310-320 mya during the carboniferous when the amniotes split into synapsids and sauropsids (Benton 2005; Shedlock & Edwards 2009; Ofstedal 2002a). Synapsids are characterized by their single temporal opening (Benton 2005). The sauropsids, on the other hand, are a group that gave rise to lizards, turtles, crocodiles, dinosaurs and the descendants of dinosaurs, birds (Ofstedal 2002a). With exclusion of birds, they are commonly referred to as reptiles and thus form a paraphyletic group. Sometimes early synapsids like the Dimetrodon (belonging to the Sphenacodontidae, see Figure 2) are referred to as mammal-like reptiles, but it is important to note that despite the name they belong to the clade Synapsida and are not “reptiles”. Through successive radiations, the synapsids gave rise first to therapsids and then

Figure 1: Diagram of the subsequent radiations within the synapsids lineage, beginning with the split between Synapsida and Sauropsida. The dotted line indicates the end-permian extinction. From Ofstedal (2002a), published in Journal of Mammary Gland Biology and Neoplasia, © Springer US.
cynodons. The latter were the dominating fauna until the dinosaurs took over in the late triassic (Oftedal 2002a). The first divide within the Mammalia took place in the late Triassic, approximately 220 mya, and split the mammalia into prototheria and theria (Madsen 2009). The egg-laying monotremes are descendants of the prototheria. Some of their characteristics like oviparity and a hairy mammary patch instead of teats are considered ancestral features of early mammals (Oftedal 2002a). The divergence of eutherians and marsupials is molecularly approximated to have occurred 176 mya in the middle Jurassic, coinciding with the split of the supercontinent Pangea into Laurasia and Gondwana (Madsen 2009). Fossil evidence usually places the divergences at a later point in time. For example, the oldest eutherian fossil found, Eomaia, dates back 125 mya and was a small shrub like creature (Ji et al. 2002). Considering that in all extant mammals a complex mammary gland is established, lactation was already fully developed before the split of monotremes and therians (or: in the last common ancestor) (Blackburn et al. 1989). The transition from oviparity to viviparity is considered to be irreversible suggesting that lactation evolved before viviparity (Hayssen & Blackburn 1985). Therefore, lactation must have evolved at least 200 mya in an egg-laying ancestor.

1.2. Form and Function of the Mammary Gland in Modern Mammals

The mammary gland may be the most important feature of mammalian reproduction. It fulfills various essential functions that are necessary for the survival and nutrition of the neonate as well as for reproductive success (Akers & Denbow 2013; Kawasaki et al. 2011). Its morphology and functions will be discussed briefly.

1.1.1. Morphology of the Mammary Gland

The basic structure of mammary glands is similar in all mammals. At the histological level, it consists of alveoli that are lined with a secretory epithelium, surrounded by myoepithelial cells. The alveoli are connected by a network of ducts and form different lobes that are drained by larger lactiferous ducts. In eutherians, these lead to cisterns - that can be very distinct in ruminants - from which milk ducts lead to one or multiple teat openings. In contrast to eutherians and marsupials, monotremes do not have a nipple but secrete milk from a hairy mammary patch (or areola) with multiple orifices (see fig. 1) (Owen 1832). This is presumed to be the ancestral condition (Oftedal 2002a; Lefèvre et al. 2010). The number and localization of mammary glands varies between species but correlates with the number of offspring in some species. The total number of glands approximates the maximal litter size whereas half of it matches the mean litter size in rodents (Gilbert 1986). The mammary gland is unique in that it is the only gland that goes through cycles of proliferation, differentiation and regression (Akers & Denbow 2013).

Figure 2: A magnified view of the mammary gland of the platypus. Top left: Mammary areola with hair removed. Right: Magnified view of one lobule. Bottom: Fully developed mammary gland. From Oftedal (2002a), original drawing by Owen (1832).
1.1.2. Nutritious and protective function

The nutritious function of the mammary gland is maybe the most obvious one and the young of all mammals rely on milk as their first source of food. Milk delivers the essential nutrients needed by the mammalian young whether the neonate is altricial or precocial (Oftedal 2012). There is much variation in the milk composition and energy density between different species, but the majority of mammalian milks contain the main macronutrients water, sugars, lipids and proteins in different concentrations (Skibiel et al. 2013). Skibiel et al. (2013) proved that this variation in milk composition depends primarily on the species phylogeny, or in other words evolutionary history. Thus, more closely related species secrete milk that is more similar in composition. After controlling for phylogeny, relative lactation length (= absolute lactation length / (absolute gestation length + absolute lactation length)) and diet have the biggest impact on milk composition (Skibiel et al. 2013). The milk composition also varies over the course of the lactation period in order to satisfy the changing needs of the young during their ontogenetic development. This is the most specialized in marsupials, where it is possible that two young of different age each receive milk different in composition from two adjacent mammary glands (asynchronous concurrent lactation) (Nicholas et al. 1995).

Though milk production seems to be a costly process, it may help to conserve energy. For example, it allows storing energy in adipose tissue or gathering food at remote locations in order to release the energy as milk at a different time or place (Grzimek 2003). On top of this the mother’s food is transformed into milk that is utilizable by the young (Peaker 2002). This buffering conserves energy because the mother does not need to make multiple trips to gather food for her young as do some birds for example (Grzimek 2003). Therefore, nutrition of the young is assured and becomes partly independent from the season or the availability of food in the proximity.

<table>
<thead>
<tr>
<th>Evolutionary strategy</th>
<th>Examples of agents in milk</th>
</tr>
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<tbody>
<tr>
<td>Directly compensate for developmental delays</td>
<td>Secretory IgA, lactoferrin, lysozyme, cytokines, PAF-acyetylhydrolase</td>
</tr>
<tr>
<td>Indirectly compensate for developmental delays</td>
<td>Oligosaccharides and nucleotides</td>
</tr>
<tr>
<td>Enhance poorly expressed functions</td>
<td>Cytokines, anti-idiotypic antibodies</td>
</tr>
<tr>
<td>Adapt the GI tract to extrauterine life</td>
<td>Epithelial growth factors</td>
</tr>
<tr>
<td>Prevent GI inflammation</td>
<td>PAF-acyetylhydrolase, anti-oxidants, IL-10</td>
</tr>
<tr>
<td>Enhanced survival of defense agents</td>
<td>Innate resistance to digestion inhibitors of proteolysis</td>
</tr>
<tr>
<td>Produce defense agents by partial digestion of substrates in milk</td>
<td>Liberation of antimicrobial fatty acids and monoglycerides from triacylglycerols</td>
</tr>
<tr>
<td>Establish commensal bacterial flora</td>
<td>Lactobacillus growth factors</td>
</tr>
</tbody>
</table>

Table 1 Evolutionary strategies compensating the delayed immune functions and protecting the mammalian young.


The nutritive function of milk is directly linked to its protective function; sugars and especially proteins are not only sources of energy and amino acids but have direct and indirect protective functions (Goldman 2002). Many of these constituents, such as the α-, β-, and κ-caseins, β-lactoglobulin, α-lactalbumin, whey acidic protein, membrane enclosed lipid droplets, lactose and oligosaccharides are unique to milk and do not occur elsewhere in nature (Oftedal 2012). In all mammals studied, there is an intra- and extra-uterine delay in the development of the immune system. Transfer of protective agents via the placenta and via milk compensate for this delay (see table 2) (Goldman 2002).
The sugars contained in milk (lactose and oligosaccharides) function as prebiotics, promoting the growth of beneficial bifidobacteria (Urashima et al. 2012). Additionally, milk oligosaccharides are similar to intestinal glycoconjugates and act as receptor analogues that prevent some pathogenic bacteria from binding to the intestinal mucosa (Goldman 2002).

Figure 3: Milk constituents and their origins. Blue: Whey proteins that are also found in mucus secretions. Red: Whey proteins derived from blood. Black: Proteins and sugars that are unique to milk. This figure shows κ-casein to be derived from fibrinogen, whereas new research (Kawasaki et al. 2011) suggests that this is probably not the case and that κ-casein evolved from the same ancestral gene as the other caseins found milk but via a different pathway (see below/later). From Vorbach (2006), published in BioEssays, © 2006 Wiley Periodicals, Inc.
The proteins contained in milk are divided into caseins that precipitate at an acidic pH and whey proteins that stay soluble. While caseins have a nutritious function supplying the offspring with calcium, phosphate and amino acids, many of the whey proteins originate from the innate and adaptive immune system and have a protective function. For example, the metal-binding proteins, lactoferrin and transferrin, show bactericidal and bacteriostatic properties (Vorbach et al., 2006). During the prepartum period, the mammary gland produces colostrum that is rich in antibodies (especially IgG), which can be absorbed by the neonate for up to 24-36 hours post partum. These immunoglobulins protect the neonate against the various and abundant environmental pathogens during the period of upbringing. Until weaning, the young are also protected by a lactogenic immunity: slgA binds to pathogens inside the intestinal lumen and respiratory tract. Remarkably, in mammals that can transfer IgG via the placenta (e.g. primates and rodents) IgA is the most common antibody in milk whereas in species that cannot transfer IgG via the placenta the predominant antibody in milk is IgG (Goldman, 2002). Many of the protective proteins present in milk are also found in other cutaneous secretions and blood where they are part of the innate immunity and adaptive immunity (Vorbach et al. 2006). This led Vorbach (2006) to propose that the mammary gland originally had a protective function and evolved from the innate immune system (see figure 3 and 2.1 Hypotheses about the primordial function).

1.1.3. Other functions
In addition to its nutritional and protective functions the mammary gland, its secretions and the suckling behavior fulfill other maybe less obvious functions that promote survival of the young (Capuco & Akers 2009). Attractant pheromones in milk promote proximity between the mother and the offspring (Graves & Duvall 1983). The relatively long presence of the mother in the beginning of the life of her young is protects the young against predation (Peaker 2002). Another idea is the concept of parental privilege according to which the prolonged proximity between parents and offspring makes the parents “part of the behavioral context of their offspring”, allowing teaching and learning (Stewart & Cohen 1997).

2. THE ORIGIN OF LACTATION AND THE MAMMARY GLAND
The mammary gland as an organ could not have evolved at once in its complexity. In order for it to evolve, there had to be an underlying developmental pattern or function from which it was derived by cooption (Oftedal 2002a) and a selective advantage gained from its earliest function. There are numerous hypotheses about the original function of the mammary glands ancestor and the tissue from which it is derived. These hypotheses are not mutually exclusive and are often speculative because they cannot be supported with enough evidence although they still offer plausible scenarios (Blackburn 1991). The different hypotheses on the origin of lactation have been reviewed in great detail by Blackburn et al. (1989; 1991) and Oftedal (2002a) but only a selection will be discussed in this review.

2.1. HYPOTHESES ABOUT THE PRIMORDIAL FUNCTION
Building on Darwin’s (1872) initial hypothesis, and those of others such as Bresslau (1907) and Gregory (1910), Long (1969; 1972) proposed the following scenario. After the evolution of a ventral
vascularized incubation patch in homoeothermic “mammal like reptiles”, cutaneous secretions provided moisture or worked as an adhesive for the eggs. Then, after the evolution of a marsupium the young started ingesting the cutaneous secretions, and subsequently the nutritious functions became more pronounced as suckling behavior, a more nutritious secretion and nipples evolved (Long 1969; 1972). Graves and Duval (1983) suggested the cooption of the secretion for pheromone signaling, attracting the young to the mother. Oral uptake to the vomeronasal organ might have facilitated the subsequent evolution of sucking behavior. Other functions that have been suggested are thermoregulation either by improving temperature transfer at the incubation patch (Bresslau 1907; Gregory 1910) or by evaporative cooling (Haldane 1965).

More recently, Oftedal (2002b) put forward the following hypothesis. The absence of fossil evidence of synapsids eggs and the study of monotreme and squamate eggs suggest that the eggshell of synapsids eggs was not mineralized. The eggs of endothermic synapsids probably had a permeable parchment-like shell, making them sensitive to desiccation when incubated. To prevent this, they either had to be kept in an incubation pouch or needed to be supplied with moisture. In a pouch the eggs would have been vulnerable to crushing and might have impeded maternal movement of predatory Triassic therapsids. Therefore, Oftedal (2002b) suggests that the eggs were supplemented with cutaneous secretions that provided moisture and allowed the predatory Triassic therapsids to leave their eggs in the nest. He further speculates that if early synapsids with glandular skin were already capable to moisturize their eggs, like existent amphibians, then they were preadapted for the development of endothermy.

Considering that calcification of the eggshell in birds and viviparity in reptiles can be interpreted as protection mechanisms against microbial pathogens, Blackburn and Hayssen (1989) identified microbial pathogens as an important selective pressure. They suggest that milk first evolved as an antimicrobial secretion protecting the eggs against microbial predation, and it was only later that acquired its nutritional function. After accidental ingestion, such a secretion might have helped control enterobacterial levels and provided additional amino acids (Hayssen & Blackburn 1985). Blackburn and Hayssen (1989) base their hypothesis on the antimicrobial properties of milk in extant mammals and on the evolutionary history of α-lactalbumin. α-lactalbumin is evolutionarily derived from c-lysozyme, an enzyme that cleaves the peptidoglycan bonds of bacterial cell-walls, indicating that the mammary gland precursor secreted lysozyme (Hayssen & Blackburn 1985). This view is also shared by Vorbach et al. (2006) who propose a scenario where the mammary gland evolved from a protective skin gland (see figure 4). In addition to the ancestry of lysozyme, they base their hypothesis on the role of xanthine-oxido-reductase (XOR) and the conserved canonical signaling pathways (Jak/Stat) that play a role in innate immunity as well as lactation. XOR plays an important role not only in the purine catabolism but also in the innate immune system, where it produces reactive oxygen species and reactive nitrogen species (Vorbach et al. 2006). In milk, XOR stabilizes the milk fat droplet membrane (Oftedal 2012).
Alternatively, analogies, such as pigeons providing their hatchlings with crop milk, suggest that lactation-like strategies can evolve with the sole purpose of feeding the young and that nourishment of the young might provide a sufficient selective advantage (Lefèvre et al. 2010).

The proposed primordial functions of a protolacteal secretion can be assigned to the following categories: 1) thermoregulation, 2) prevention of desiccation, 3) promotion of proximity between mother and eggs through an adhesive secretion or by pheromone signaling towards the offspring, 4) protection against microbial pathogens and 5) nutrition of the young. Although, most authors consider the nutritive function to have evolved subsequently to the other proposed functions.

![Figure 4: Proposed scenario of the evolution from an epithelial gland that secreted antimicrobial enzymes to a mammary gland with a nutritious secretion.](image)

Figure 4: Proposed scenario of the evolution from an epithelial gland that secreted antimicrobial enzymes to a mammary gland with a nutritious secretion. Lysozyme and xanthine oxidoreductase (XOR) both have antimicrobial properties and are part of the innate immune system. Through gene duplication, α-lactalbumin evolved from lysozyme. α-lactalbumin together with β-1,4 galactosyltransferase forms the lactase synthetase complex. XOR acquired an important role in the stabilization of the milk fat globule by gene sharing. From Vorbach (2006), published in BioEssays, © 2006 Wiley Periodicals, Inc.

### 2.2. Hypotheses about the tissue origin

As for its primordial function, there are also different hypotheses on the original tissue or the type of gland from which the mammary gland is derived. Evolutionary scenarios that have been suggested are:

1) the *de novo* evolution,
2) the evolution from one of the main types of skin glands (eccrine, apocrine or sebaceous),
3) the evolution from an ancestral gland or gland complex (Oftedal & Dhouailly 2013) from which also the above mentioned types of gland are derived, and

4) a mosaic gland with apocrine as well as sebaceous properties (Blackburn 1991).

The idea of a de novo evolution of the mammary gland seems unlikely, lacks evidence and "is an explanation of last resort" according to Blackburn (1989). In contrast to the de novo evolution, a gradual evolution of the mammary gland by cooption of an underlying structure seems more likely and similarities between mammalian skin glands and the mammary gland indicate this (Oftedal 2002a). Additionally, recent genetic studies by Lemay et al. (2009) also suggest that the mammary gland evolved by "co-opting existing structures and developmental pathways" (Lemay et al. 2009). The fossilized integument of Permian therapsids indicates that therapsids had alveolar skin glands (Chudinov 1968; Oftedal 2002a). They inherited this glandular skin from tetrapods ancestors and glandular integument remains a feature of mammals to this day (Chudinov 1968; Oftedal 2002a). Skin glands in mammals are usually classified according to their mode of secretion and the three main types are eccrine, apocrine and sebaceous glands. The eccrine glands secretions are watery and the secretory products are released by exocytosis without loss of cytoplasm or plasma membrane (merocrine secretion). Apocrine glands can additionally secrete their products by membrane budding (apocrine secretion), as is the case in the secretion of the milk fat globules (Oftedal 2002a). Sebaceous glands release their sebum though a holocrine process, in which cells disintegrate releasing their products. When compared, the mammary gland shares many features with apocrine glands and some with sebaceous glands. The most striking similarities (see also table 2) are 1) the bilayered secretory part consisting of a secretory epithelium surrounded by myoepithelial cells, 2) the secretion via both merocrine and apocrine ways, 3) the associations with hair follicles and sebaceous glands and 4) the need for hormonal maturation (Oftedal 2002a; Oftedal & Dhouailly 2013).

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<th>Apocrine gland</th>
<th>Sebaceous gland</th>
<th>Mammary gland</th>
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<tbody>
<tr>
<td>Embryonic origin</td>
<td>Apo-pilo-sebaceous anlagen</td>
<td>(Apo)-pilo-sebaceous anlagen</td>
<td>Mammo-pilo-sebaceous anlagen</td>
</tr>
<tr>
<td>Secretory epithelium</td>
<td>single layer</td>
<td>multiple layers</td>
<td>single layer</td>
</tr>
<tr>
<td>Mode of secretion</td>
<td>apocrine, merocrine</td>
<td>holocrine</td>
<td>apocrine, merocrine</td>
</tr>
<tr>
<td>Myoepithelial cell layer</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Prolactin responsive</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Androgen responsive</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Motor innervation</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Stage of maturation</td>
<td>puberty</td>
<td>puberty</td>
<td>estrus or pregnancy</td>
</tr>
</tbody>
</table>

Table 2: Comparison of some characteristics of apocrine, sebaceous, and mammary glands. Compiled with information from Blackburn (1991) and Oftedal (2002a).
Blackburn (1991) suggests that “a new structure need not evolve from a single phenotypic precursor, as long as an organism has the genetic and developmental potential to recombine pre-existing components into novel combinations”. Even though the mammary gland shares many characteristics with apocrine glands, it lacks motor innervation and androgen responsiveness while it is responsive to prolactin. He further argues that if the mammary gland had evolved from an apocrine gland it must have lost the first two functions and there must have been a convergent evolution of prolactin responsiveness in the mammary gland and sebaceous glands. Therefore Blackburn (1991) proposed that "the mammary gland may represent a neomorphic hybrid, a mosaic organ whose evolution involved the incorporation of characteristics coded for in the genome, but expressed differently by separate populations of skin glands."

Oftedal (2002a) disputed Blackburn’s theory because he doubts that the lost features are typical for apocrine glands. Furthermore it is unlikely that the “apocrine glands remained unchanged since milk secretion first appeared among synapsids”(Oftedal 2002a). Many similarities between apocrine and mammary glands and the little resemblance between mammary glands and sebaceous glands point to a relationship between apocrine and mammary glands. Oftedal (2002a) therefore proposed that the mammary gland evolved from an ancestral apocrine-like gland that is the precursor of mammary glands and current apocrine glands. Notably apocrine glands are usually associated with a hair follicle and a sebaceous gland, forming a triad called apo-pilo-sebaceous unit (APSU) (Oftedal, 2012). As mentioned earlier monotremes have a hairy mammary patch and their mammary glands are associated with hair and a sebaceous gland. In analogy to the APSU, this triad is called mammo(lobular)-pilo-sebaceous unit (MPSU). The MPSU is assumed to be the ancestral to modern mammals but in marsupials and eutherians the association of mammary glands and hair follicles has been lost whereas apocrine glands are still associated with hair follicles (Oftedal 2002a). Oftedal (2002a) speculated that this ancestral condition must be inhibited in eutherians, and new research suggests that this might be the case. Mayer et al. (2008) showed that bone morphogenetic protein inhibits hair follicle development and that mice with deficient BMP (by transgenic over expression of a BMP antagonist) had pilo-sebaceous units in the nipple epithelium. During the evolution of the nipple the BMP pathway might have been co-opted in order to suppress hair follicle development (Mayer et al. 2008). This probably happened after the parting of marsupials and eutherians because the development of hair follicles is not suppressed in marsupials but the hair is shed later on (Oftedal 2012; Mayer et al. 2008).

To further investigate the connection between the APSU and the MPSU, Oftedal and Dhouailly (2013) compared the embryological origin and morphogenesis of the mammary gland in monotremes, marsupials and eutherians. According to them, most mammals share the same phases of early mammary gland development. These seven phases, the development of 1) mammary lines, 2) mammary placodes, 3) mammary bulbs, 4) primary sprouts, 5) secondary sprouts, 6) sprout canalization and 7) terminal branching, occur in most mammals studied. Oftedal and Dhouailly (2013) found that in monotremes 100-200 primary sprouts develop from a plate-like mammary bulb and at the end of each primary sprout a fully functional MPSU develops. The MPSU-triad consists of a mammary gland lobule and a sebaceous gland that both open into the infundibulum of the mammary hair follicle.
In marsupials a single primary sprout develops per mammary gland. Afterwards a MPSU is formed, the sprout is hollowed out and a nipple emerges while the mammary hairs are shed. At least in some eutherians a MPSU occurs transiently during development but the development of hair is probably inhibited by the earlier mentioned pathway. This suggests that the MPSU is ancestral to the three clades of modern mammals and that it is derived from an ancestral APSU-MPSU that was incorporated into the evolving mammary gland (see figure 5). When this ancestral APSU-MPSU evolved is not known and it might be important to note that only the mammary ductal tree and secretory tissue are derived from it. Other structures such as mammary lines, mammary placodes and their derivates have a different origin (Oftedal & Dhouailly 2013).

Figure 5: Illustration of the possible evolution of the mammary gland (mammolobular-pilo-sebaceous unit) and the apo-pilo-sebaceous unit from an ancestral APSU-MPSU. From Oftedal (2013), published in Journal of Mammary Gland Biology and Neoplasia, © Springer US. Color key: dark green = mammary secretory cells, cyan = sebaceous gland, seagreen = apocrine gland, dark cyan = apocrine- mammary transitional cells

3. OTHER EVIDENCE FOR THE EVOLUTION OF THE MAMMARY GLAND

3.1. PALEONTOLOGICAL APPROACH

Because of its soft tissue, no direct evidence of mammary glands has been found in the fossils of early mammals (Oftedal 2002a). Therefore, there is no hard evidence in the fossil record about when the mammary gland evolved. However there are indications for typical mammalian characteristics that are associated with lactation:
1) A fully developed secondary palate made breathing while feeding possible and in combination with fleshy cheeks might have allowed suckling (Benton 2005; Oftedal 2002a).

2) Diphyodonty in late cynodonts suggests that the young relied on milk. Milk can even be considered a prerequisite for the evolution of diphyodonty (Pond 1977; Oftedal 2012; Benton 2005).

3) Epipubic bones might have supported a pouch in which eggs or suckling offspring were transported (Oftedal 2002a) but also have a locomotor function (Benton 2005).

4) The evidence of hair comes from the earliest found eutherian fossil, Eomaia 125 mya (Ji et al. 2002), but at this time lactation had already been established.

5) Concave structures in the fossilized skin of Permian therapsids have been interpreted as an indication for glandular skin (Chudinov 1968; Oftedal 2002a).

6) Extensive and more uniform bone and tooth development in dependent young required calcium supplementation (Capuco & Akers 2009), possibly with caseins (Kawasaki et al. 2011).

7) Respiratory turbinals permit moisture and heat exchange and are indicative for endothermy and increased metabolic rates (Oftedal 2002a). Endothermy required the incubation of the eggs and the increased energy demand required supplementation of the hatchlings food, possibly met with milk (Hopson 1973).

3.2. GENETIC AND MOLECULAR APPROACHES

3.2.1. The lactation genome

With the bovine lactation genome at hand, Lemay et al. (2009) compared it to the genomes of monotremes, marsupials and a eutherian. They found that there is a higher conservation of milk and mammary genes and that in the bovine genome these genes are more likely to have undergone duplication since the last common ancestor with the platypus. The species-specific variations in milk composition seem to be caused by variations in copy number rather than changes in the gene sequence. Furthermore, milk and mammary genes are under strong negative selection (stabilizing selection) and no positive selection (diversifying selection). This means that the evolution of lactation is restricted in order to maximize both survival of the mother and survival of the offspring. The most divergent proteins in the lactation genome have nutritional and immunological functions. This can be explained by a continuing selection of these genes as a reaction to different nutritional and pathogen challenges in different environments and reproductive strategies (Lemay et al. 2009).

3.2.2. Milk constituents and evolution

3.2.2.1. Evolution of caseins

The caseins belong to the secretory calcium-binding phosphoprotein family and make up the largest fraction of milk proteins. The caseins are essential for the assembly of casein micelles that supply the young with calcium and phosphate needed for bone growth and cellular metabolism. At the same time they are an important source of amino acids (Lefèvre et al., 2009; Kawasaki et al., 2011). There are two groups of caseins in milk: the calcium sensitive caseins (α- and β-caseins) and the not-calcium sensitive caseins (κ-casein). The first bind calcium and precipitate at high calcium concentrations...
whereas the calcium insensitive κ-casein plays an important role in stabilizing the micelle (Kawasaki et al. 2011). Once ingested, digestive proteases break down κ-caseins and destabilize the micelle. The precipitated caseins form a gastric curd that traps lipids that can now be broken down by lipases. The evolution of this mechanisms was essential for efficient digestion of liquid mammary gland secretions (Oftedal 2013).

![Figure 6: Simplified representation of the evolution of casein genes via two pathways from one ancestral gene (ODAM) that was already present in early tetrapods and gave rise to the different casein genes by repeated duplications. Based on (Kawasaki et al. 2011)](image)

At first it was suggested that the CSN1/2 casein genes that code for the production of α- and β-caseins evolved at about the time of the division between therians and monotremes (Kawasaki & Weiss 2003). But Brawand et al. (2008) found three possible casein genes in the platypus that lie in syntenic genomic regions in therians. Additionally they found an orthologous gene of the k-casein gene (CSN3) that is not homologous with the other caseins. This might indicate that caseins in genes were already in existence in the common mammalian ancestor (Brawand et al., 2008). According to Brawand et al. (2008) mammalian ancestors already had caseins 310 to 200 mya. Kawasaki et al. (2011) suggest that all casein genes evolved from the odontogenic ameloblast-associated (ODAM) gene by repeated duplications via two different pathways (see figure 6). They further suspect that caseins (CSN1/2) might have evolved in amniotes before the split of synapsids and sauropsids 310 mya. In early protolacteal secretions caseins probably inhibited spontaneous calcium phosphate precipitation, facilitating calcium phosphate passage through the eggshell. When milk evolved to include higher calcium concentrations caseins also evolved to adapt to the need to further stabilize the casein micelle (Kawasaki et al. 2011).
Caseins have a similar function in milk as vitellogenin in egg. Because of the rise of lactation and the possibility of casein to take over functions of vitellogenin, vitellogenin became more and more dispensable (Brawand et al. 2008). Thus, there was a reduction of the negative selective pressure for the production of yolk. This might explain the gradual loss of function of VIT genes from originally three to one in the platypus and finally to zero functional VIT genes in theria. The disappearance of VIT genes correlates with a reduction in yolk and egg size in the platypus. Therefore lactation might be the prerequisite for the loss of VIT genes. In marsupials the last remaining VIT gen (VIT2) might have become abundant because of their more specialized lactation whereas in eutherians the placenta made the production of yolk unnecessary (Brawand et al., 2008).

3.2.2.2. Evolution of milk sugar synthesis

All mammalian milks contain carbohydrates in varying concentrations (up to 10%) (Urashima et al. 2012). In eutherian milk the predominant sugar is the disaccharide lactose whereas oligosaccharides predominate in marsupial and monotreme milk (Urashima et al. 2012). Lactose and milk oligosaccharides are unique molecules not occurring elsewhere in nature. This limits the bacteria colonizing milk secretions to beneficial bifidobacteria that can break down lactose (Urashima et al. 2012). Additionally, oligosaccharides act as receptor analogues for pathogenic microorganisms (Urashima et al. 2012; Goldman et al. 1998) The milk oligosaccharides are produced from lactose by glycosyl transferases and lactose is produced by the heterodimer lactose synthetase complex that consists of α-lactalbumin and β1,4-galactosyltransferase (Urashima et al. 2012). α-lactalbumin regulates the rate at which lactose is synthesized and is of particular interest because it arose via gene duplication from c-lysozyme, a bacteriolytic enzyme also found in hen egg white (Hayssen & Blackburn 1985; Qasba & Kumar 1997). Hayssen and Blackburn (1985) suggested that the evolution of α-lactalbumin from lysozyme happened via an intermediate form that had both functions - bacteriolysis and lactose synthesis. Monotreme α-lactalbumins represent another intermediate step between the bifunctional protein and the common α-lactalbumins (see figure 7) (Urashima et al. 2012). Urashima et al. (2012) hypothesized that α-lactalbumin concentrations were initially low, so that little lactose was produced that was directly converted to antimicrobial oligosaccharides. Molecular data places the divergence of lysozyme and α-lactalbumin 300 to 400 mya around the split between synapsids and sauropsids (Qasba & Kumar 1997; Urashima et al. 2012). Together these findings support the scenario that protolacteal secretion had initially had antimicrobial functions as suggested by Hayssen & Blackburn (1985) and Vorbach et al. ( 2006). The latest development in milk sugar synthesis is the increased production of lactose as an important

Figure 7: Molecular evolution from lysozyme to α-lactalbumin via an intermediate bifunctional protein. From (Urashima et al. 2012), published in Animal, © The Animal Consortium.
energy source in eutherians, which depended on the prior evolution of intestinal lactase (Urashima et al. 2012).

**DISCUSSION**

Even though some of the hypotheses on mammary gland evolution are conflicting, there is a consensus amongst most authors that the evolution of lactation and the mammary gland was a gradual process and that lactation arose by co-opting existing structures and pathways. Together they create a bigger picture and a rough timeline of mammary gland evolution. When synapsids diverged from sauropsids 310 mya they inherited a glandular skin from their amniote ancestors (Oftedal 2002a; Chudinov 1968). Around this time some proteins that would later play integral roles in milk, like casein and α-lactalbumin, had probably already evolved (Kawasaki et al. 2011; Urashima et al. 2012). This suggests that early synapsids were already preadapted for the evolution of lactation (Oftedal 2002a). From the Triassic onward the mammary gland evolved while the mammalian ancestors acquired more and more mammalian characteristics. From the different hypotheses about the original function of lactation, Oftedal's scenario (2002b) of an egg supplement preventing dehydration of parchment shelled eggs and Blackburn's (1985) and Vorbach's (2006) suggestion that the primordial function was antimicrobial protection of eggs seem the most plausible. Maybe these functions developed parallel and in combination with other functions such as pheromone signaling (Graves & Duvall 1983). The latter gives a good explanation for the origin of suckling behavior. At the moment it is not possible to say which of these primitive functions came first. Most authors agree that the nutritious function of milk evolved subsequently to a primordial function. If it is correct that proto-lacteal secretions not only moisturized synapsid parchment-shelled eggs but also transferred minerals such as calcium phosphate as Oftedal (2002b) and Kawasaki (2011) suggested, than the step from a protolacteal secretion to a nutritious secretion ingested by hatchlings is small. The most primitive, as in having many ancestral features, living mammals are the monotremes. They still lay eggs with a parchment-like shell, but as Oftedal (2002b) pointed it still needs to be demonstrated that they lactate on their eggs and that the nutrients pass through the eggshell. This needs to be investigated and would give further credibility to the proposed scenario. When it comes to the tissue origin, the hypothesis of a shared ancestral APSU-MPSU from which both apocrine glands and mammary glands evolved (Oftedal & Dhouailly 2013) is convincing and is supported by the early ontogenesis of the mammary gland in modern mammals. The secretion mechanisms also point to a relatedness between apocrine and mammary glands. In order to investigate this relationship further it would be interesting to compare gene expression in apocrine glands and mammary glands similar to the comparison of milk and lactation genes by Lemay et al. (2009).

Returning to the questions put forward in the introduction, the scenarios above can explain from which underlying gland structure the mammary gland might have evolved and what its original function might have been. The time period in which it evolved is more difficult to determine. The fact that all of today’s mammals possess mammary glands indicates that lactation, was already established in their last common ancestor (Lefèvre et al. 2010). Thus, the mammary gland evolved in the time span between
the divergence of synapsids and sauropsids 310 mya and the divergence of monotremes and therians 220 mya. However, evolutionary evidence of some milk proteins dates back even further. The question of how the mammary gland evolved during this time can not be answered yet because of the lack of fossilized mammary glands. The indications for the evolution of other mammalian traits are too vague to make assertions about the developing mammary gland.

Recent research focused on the genetic and molecular evidence for mammary gland evolution. Lemay et al, (2009) compared the milk and mammary genes of modern mammals and showed that mammary gland evolution is restricted in order to maximize mother and offspring survival. This shows how essential lactation has become for mammals. The investigation of the evolutionary history of important milk proteins such as casein (Kawasaki et al. 2011) and α-lactalbumin (Urashima et al. 2012; Qasba & Kumar 1997) has revealed that the evolutionary origins of lactation date back further than assumed, preceding the divergence of Synapsida and Sauropsida.

In this review only some of the hypotheses and evidence concerning mammary gland evolution were discussed. Interesting subjects that were not discussed but might provide insights into mammary gland evolution are messaging pathways and the hormonal control of lactation. The latter might also provide answers to the question why lactation evolved to almost exclusively, with the exception of some Old World fruit bats (Kunz & Hosken 2009), occur in females.
REFERENCES


Other resources:


URL: http://www.stratigraphy.org/ICSchart/ChronostratChart2015-01.pdf (last referenced on 03.08.2015)