Investigations on the Role of the Immune System In Mammary Development And Maternal Immunity In the Marsupial, Monodelphis domestica

Bethaney Fehrenkamp

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Investigations on the Role of the Immune System In Mammary Development And Maternal Immunity In the Marsupial, Monodelphis domestica

By

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M.S., Community Health, University of New Mexico, 2012

DISSERTATION
Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy
Biology

The University of New Mexico
Albuquerque, New Mexico

December 2018
DEDICATION

This work is dedicated to my remarkable husband Brad. Without you I wouldn’t be where or who I am today. You continually encouraged me to go back to school and find exactly what makes me happy. You picked up my slack when research and writing took over our life. You listened to my late night rambles on immunology, experimentation and manuscript layout. You consoled me and encouraged me to continue this journey. Words cannot express what you mean to me. I am forever grateful.

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Abstract

All newborn mammals are highly dependent upon milk for nourishment and immune protection. This is especially true for marsupials, a lineage of mammals with a short gestation, limited placental development, and an increased reliance on an extended lactation period. Most newborn marsupials do not receive passive maternal immunity *in utero* and therefore are entirely dependent upon factors within the milk for immune protection until capable of mounting their own response. In this project we seek to characterize the complex lactation program utilized by marsupials, and seek greater understanding of the maternal role in the establishment of the developing immune system of a model marsupial, the gray short-tailed opossum, *Monodelphis domestica*. The ontogeny of opossum immune development has been well established, however investigations into correlations with changes in the mammarys during lactation are lacking in this species. Towards these goals, a combination of histology, immunohistochemistry and quantitative PCR was used to investigate mammary development and immune system presence in the opossum mammarys throughout lactation. These investigations have the potential to impact the understanding of the role the immune system plays in mammary development and function from an evolutionary perspective.
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Chapter 1

Introduction to Mammary Gland Evolution, Marsupial Lactation, and Immunobiology of Lactation

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Evolution, Structure, and Functions of the Mammary Gland

Mammary glands are a defining characteristic of mammals. Indeed, the naming of the class *Mammalia* by Linneaus (1758) refers directly to the presence of mammary glands and the use of lactation. Lactation is a complex biological process that serves to provide a continuous supply of nutritional and immunological support to developing offspring. This specialized process evolved in the synapsids and is dependent upon the evolution of a specialized structure, the mammary gland (Oftedal 2012) (Figure 1).

![Phylogenetic relationship of the extant mammalian lineages.](image)

**Figure 1: Phylogenetic relationship of the extant mammalian lineages.**
The presence of mammary glands and the use of lactation is seen throughout the mammalian lineage. Eutherian and Metatherians lineages are viviparous.
Branch length not intended to represent distance. MYA is Million years ago.
Adapted from Beninda-Emonds *et al.*, 2007; Oftedal 2012

Mammary gland structure is similar to that of skin glands, but pinpointing the exact type of ancestral gland from which it has evolved has been controversial and restricted to examination of extant lineages (Blackburn *et al.*, 1989; Blackburn 1993; Oftedal 2002). Blackburn originally suggested the mammary gland evolved from a combination of different skin gland populations into a new functional complex (Blackburn, 1993). However, more recent analyses suggest an apocrine-like gland closely associated with hair follicles as the likely evolutionary origin (Oftedal 2002; Lefevre *et al.*, 2010; Oftedal 2012). The association with hair follicles can be seen within the monotreme and marsupial lineages. In eutherians this association has been lost, but there is inhibition of hair follicle development in the mammary area, suggesting acquisition of an active
repressive mechanism within the lineage (Howard and Gusterson 2000; Oftedal, 2002). The exact pathway of mammary gland evolution remains somewhat speculative. It is clear that despite accurate timing of emergence or ancestral gland of origin, the use of the mammary gland has provided a unique reproductive strategy among all mammalian lineages. Comparative analyses are necessary in understanding the evolution of the mammary gland.

Monotremes are the extant mammalian lineage that has retained the most ancient characteristics (Figure 1). They comprise the most distantly related lineage to true placental mammals (eutherians) and are believed to retain features of early mammals that may have been lost through evolutionary time (Griffiths 1978; Oftedal, 2002). Monotremes are oviparous (egg-laying); and provide neonates with a milk secretion from mammary glands that lack an organized nipple. Rather the young lap up the milk. The ducts open directly in the areola, and the ventral area of the skin where secretion occurs is associated with a hair follicle (Griffiths 1978). This provides an example of extant transition animals, which retain the egg-laying placent-a-less nature of reproduction of reptiles and birds, with early mammary development and incorporation of milk as a provisioning strategy. Interestingly, monotreme mammary gland structure is similar across the extant species regardless of terrestrial or amphibious living (Griffiths et al., 1969, 1973; Musser 2005). The alveoli and duct system of active late stage monotreme mammarys are similar to that of active eutherian mammarys (see later description), but the areolae where the milk emerges are concealed by fur and there are no true nipples or teats (Griffiths et al. 1969; 1973). Monotreme mammary glands represent the earliest known attempt to produce milk by a sophisticated mammary gland structure (Griffiths et al. 1969; 1973; Oftedal 2002).

Like monotremes, marsupial young are born in a highly altricial state. Marsupials are viviparous, yet due to limited invasiveness of the placenta and short gestational time, offspring are extremely reliant on a complex and changing lactation program to support this external gestational strategy (Trott et al., 2003; Daly et al., 2007; Joss et al., 2009). Limited prior investigations into marsupial mammary development report similar
structures as eutherian mammary glands late in lactation with less development occurring during pregnancy (Findlay 1982; Nicholas 1988). The marsupial lineage represents the emergence of a true nipple or teat development among the extant lineages. Teats develop embryonically from epithelial stem cells concurrent with mammary differentiation (see further discussion) (Oftedal, 2002).

Among the eutherian lineage a more invasive placenta has evolved allowing for strategies that incorporate greater transplacental transfer of immune factors. This has allowed for a diversification of the role of lactation in the reproductive strategies (Jenness and Sloan, 1970). Eutherian mammals invest approximately equal maternal time into gestation and lactation (Hayssen, 1993). Unlike that of marsupials, eutherian milk composition remains relatively consistent throughout lactation, excepting for colostrum in early lactation (Hurley and Thiel, 2011, Lefevre et al., 2010). Within the eutherian lineage the most notable evolutionary change is regarding physiological placement of the mammary glands, most notable within the primate lineage. Primates have two mammary glands located in the pectoral region (Cline, 2007). This is the only lineage where a shift in mammary placement from ventral abdominal to pectoral, along with restriction of teat number, is seen throughout the entire lineage. Examples of shifting mammary placement to a more pectoral placement can be seen within some species of other eutherian lineages, suggesting convergent evolution outside of the primate lineage.

There are three main stages of mammary gland development in mammals: embryonic, pubertal and adult (Watson and Khaled, 2008). These have been most studied within the eutherian lineage and less is known regarding marsupial and monotreme lineages. In the developing eutherian embryo there are two cellular compartments of the mammary gland, the epithelial compartment and the surrounding stromal compartment. Embryologically these tissues are derived from ectoderm and mesoderm, respectively. The embryonic mammary mesenchyme provides key inductive signals that specify mammary epithelial cell differentiation. Once the mammary line has been delineated, signals from the mesenchyme regulate the dorsal/ventral patterning of placodes (Macias and Hinck, 2012). The placodes descend into the underlying mesenchyme and produce the basic ductal

Puberty initiates branching morphogenesis to create a ductal tree that fills the fat pad. During pregnancy the combined actions of progesterone and prolactin generate alveoli, which secrete and store milk during lactation (Figure 2). Terminal differentiation of the mammary gland does not occur until the first pregnancy in both eutherians and marsupials (Hinds 1988; Cline, 2007). Lack of demand for milk at weaning is believed to initiate the process of involution whereby the gland is remodeled back to its pre-pregnancy state (Cline 2007). It is only during active lactogenesis that the mammary gland takes on a secretory role. In this state in eutherians the glandular tissue occupies the majority of the mammary fat pad (Figure 2) (Cline, 2007).

**Figure 2: Histological section of active eutherian mammary gland.**
20X magnification of H&E stained active murine mammary glands one day postpartum. Secretory alveoli occupy the majority of the fat pad and branched ducting structures are evident. Note the grape like clusters of enlarged alveoli for milk production and storage. Histobank #1587 ucdavis.edu

The primary role of the mammary gland is to serve as a secretory organ for milk production and delivery. Morphological restructuring initiated with pregnancy in eutherians includes an increase in secondary and tertiary ductal branching, providing a ductal network for alveolar development. Proliferating epithelial cells generate alveolar buds that progressively cleave and differentiate into distinct alveoli, which become milk-
secreting lobules during lactation (Watson and Khaled, 2008; Macias and Hinck, 2012). Interstitial adipose tissue recedes as the proliferating epithelial cells occupy the interductal spaces (Figure 2). Increased vascularization occurs by mid-pregnancy in eutherians, and each alveoli is surrounded by a network of capillaries (Djonov et al., 2001). By late pregnancy the alveoli encompass the majority of the fat pad and some secretory activity has been noted as pregnancy approaches term.

In addition to their defensive roles, cells of the immune system have also been found to participate in eutherian mammary development and remodeling (reviewed in Reed and Schwertfeger, 2010). In particular, eosinophils are crucial in mammary development (Gouon-Evan et al., 2002; Reed and Schwertfeger, 2010; Beaudry et al., 2016). One of the cytokines known to play a role in mammary immune cell trafficking in eutherians is interleukin (IL)-16 (Böttcher et al., 1999). IL-16 produced by eutherian mammary epithelial cells during lactation is chemotactic for CD4^+ T cells, monocytes, and eosinophils (Cruikshank et al., 1987; Rand et al., 1991; Chollet-Hinton et al., 2014). To date no studies have reported on leukocyte infiltration at any stage of marsupial mammary development. A limited number of studies have reported on cellular presence within the milk secretions, however these were limited to microscopic identification and did not further characterize cell populations (Young et al., 1997; Young and Deane 2001). It is unknown whether the presence of leukocytes within developing mammarys is conserved across the lineages or is eutherian specific.

The nutritional provisioning component of lactation is related to macromolecule secretion by the mammary glands. Across mammalian species, substantial variation in macromolecule composition (protein, fat, and carbohydrates) of milk exists (Jenness and Sloan, 1970, Lefevre et al., 2010, Skibiel, et al., 2013). Despite the interspecies variation in milk composition, there is typically little variation in the milk the offspring receives throughout the course of development as milk composition remains relatively consistent over the course of lactation (Jenness and Sloan, 1970). On the contrary, marsupials utilize one of the most sophisticated lactation programs of all mammals, involving a changing
composition of milk, both nutritionally and immunologically, throughout lactation to further support the developing offspring (Green and Merchant, 1988; Joss et al., 2009).

**Marsupial Lactation**

Dependence on milk for both nutritional and immunological provisioning is most pronounced in marsupials, a lineage of mammals noted for a shortened gestation and giving birth to highly altricial young (Tyndale-Biscoe and Renfree, 1987). This external gestation strategy requires the offspring to complete its “fetal” development outside the protection of the womb typically in a pouch structure. Marsupials are born immunologically immature, creating an intriguing conundrum related to how offspring manage to complete their development in the external environment surrounded by pathogens without the benefit of a functional adaptive immune system (Tyndale-Biscoe and Janssens, 1988; Old and Deane 2000; Parra et al. 2009; Wang et al. 2012). To support the continued development of the offspring a complex and changing lactation program has co-evolved within the mammaries (Green and Merchant, 1988; Joss et al., 2009).

Appreciation for the complexities of marsupial lactation first appeared in the literature in 1833, but the first characterization of marsupial milk components was not reported for another 120 years (Morgan, 1833a; 1833b; Collie, 1833; Bolliger and Pascoe, 1953). Changes in milk composition throughout lactation in a marsupial were first characterized in the Australian wallaroo (*Macropus robustus*) (Bolliger and Pascoe, 1953). Across all marsupial species studied to date, a change in nutritional composition of milk throughout lactation has been noted, in contrast to consistent composition seen in eutherian mammals (Green and Merchant, 1988). Early in marsupial lactation the milk is dilute and contains low levels of proteins and fats and high concentrations of carbohydrates, before transitioning to a higher concentration of proteins and fats with little carbohydrates in late lactation (Bolliger and Pascoe, 1953; Gross and Bolliger, 1959; Lemon and Barker, 1966; Griffiths et al., 1972; Messer and Green, 1979; Green et al., 1980; Lincoln and Renfree,
Some macropod species of marsupials, such as the Australian tammar wallaby (*Macropus eugenii*), engage in a highly complex behavior of nursing two offspring of differing developmental ages simultaneously (Nicholas *et al.*, 1988). This involves one mammary gland producing milk of late stage composition while an adjacent gland produces milk of early stage composition. This phenomenon is referred to as asynchronous concurrent lactation (ACL) and appears to be a unique adaptation of lactation related to embryonic diapause, or the process of a fertilized embryo remaining in a quiescent state for a period of time before restarting development, that is not seen outside the marsupial lineage (Lincoln and Renfree, 1981; Tyndale-Biscoe and Janssens, 1988). ACL demonstrates the importance of local control of the lactation program including shifts in macromolecule composition as well as stage-specific expression of major milk proteins.

Jenness *et al.*, (1964) was the first to suggest that the offspring suckling behavior may be an influencing factor on the changing nutritional composition of marsupial milk. Simple, yet elegant fostering experiments have since shown the dynamics of lactation to be a true genetic program controlling milk composition irrespective of strength or duration of suckling stimulus of the developing offspring (Trott *et al.*, 2003). It is apparent that suckling stimulus is required to continue lactation beyond parturition, but is not sufficient to change the composition of the milk in subsequent stages. These fostering experiments also demonstrated the evolutionary association of milk composition and offspring development as perturbed development resulted from consumption of milk from an incorrect stage (Trott *et al.* 2003; Kwek *et al.*, 2009; Menzies 2013). Marsupial lactation is indeed more complex than that of eutherians evidenced by a dynamic lactation scheme of altering milk composition to suit the developmental needs of the offspring.
The varied milk composition as well as changing behavior and development of the offspring led to defining phases of lactation in marsupials. For purposes of discussion phases will be described here within the tammar wallaby (Macropus eugenii), as this has become a model species for marsupial research and the majority of what is known regarding marsupial lactation is concerning this species (Nicholas, et al., 1997). M. eugenii lactation has been divided into three phases (Figure 3). The first phase includes a shortened gestation followed by parturition and lactogenesis. This is similar to other mammalian species where the mammary glands are preparing for the production of milk. During phase 1 all four teats of the dam will begin to develop in preparation for parturition. Following parturition the single wallaby offspring will crawl into the pouch and attach to a teat. The remaining non-utilized teats will enter a quiescent stage and only the mammary gland being suckled will continue through the subsequent phases. Phase 2 begins at parturition and is further subdivided (2A and 2B) corresponding to obligate attachment of offspring to the teat. Phase 2A is approximately 100-120 days in duration in the wallaby and is characterized by the permanent teat attachment of the offspring and a production of milk that is dilute with a low concentration of proteins and fats and a high concentration of carbohydrates. The period of permanent attachment has been likened to the later part of intrauterine gestation in eutherian mammals (Sharman, 1970).

Figure 3: Phases of Lactation in a tammar wallaby (Macropus eugenii)
Phases of lactation have been defined in the wallaby based on offspring behavior and milk composition. Adapted from Nicholas et al., 1997
After approximately 100 days the offspring will release the teat, yet remain in the pouch and will suckle intermittently, more like the suckling behavior of eutherian neonates, for an additional 100 days (2B). The composition of the milk remains relatively constant throughout phase 2, also referred to as early lactation. Phase 3 is considered late lactation and is defined by the wallaby offspring’s evacuation of the pouch (around 200 days post-partum). An increase in milk quantity as well as a change in composition to a high protein, high fat, low carbohydrate milk is produced in late lactation (Messer and Green, 1979; Green et al., 1980; Green et al., 1983; Joss et al., 2009) This late lactation milk serves as a supplement to the offspring as it prepares to wean at 350 days post-partum (Figure 3).

Phases have been further defined by the changes in expression levels for a number of milk protein genes described in several Australian marsupial species, in particular lactation stage-specific genes such as early lactation protein (ELP), mid-late whey acidic protein (WAP) and late-lactation proteins (LLP-A and LLP-B) (Nicholas et al., 1987; Joss et al., 2007). With the exception of WAP, which is also found in the milk of many eutherians excluding human, goat and sheep, these phase-specific milk proteins are marsupial specific and have not been found in Eutherian or monotreme milk (Hajjoubi et al. 2006; Joss et al., 2007). An additional marsupial specific lactation protein Very Early Lactation Protein (VELP) has also been detected in T. vulpecula and M. eugenii prior to the detection of ELP (Kuy et al., 2007; Joss et al., 2007). The protein sequence of VELP appears similar to Mannose Binding Lectin (MBL) and therefore has been speculated to play a role in activating the complement system within the neonate (Joss et al., 2009).

These phases of marsupial lactation have been proposed to be universal within the marsupial lineage, however investigations have focused on M. eugenii with limited investigations in other species, including American marsupials.
Evolution of Passive Immunity

Offspring are generally born with a naïve adaptive immune system leaving them more vulnerable to infection and death than adults. In vertebrates, providing early immunological protection to offspring is achieved by a variety of strategies of incorporating maternally derived immune factors, commonly in the form of antibodies, into the reproductive process. Two main strategies to transfer maternally derived antibodies emerge throughout vertebrates. Loosely these can be considered “pre” and “post”-natal strategies. The emergence of ‘pre-natal strategies’ appear within some viviparous sharks, teleost fish, amphibians, reptiles and birds, which have been shown to provide antibodies to offspring via the yolk sac (West et al., 2004; Haines et al., 2005; Swain and Nayak, 2009). Early use of ‘post-birth strategies’ also appears in some birds, which have been shown to provide maternally derived antibodies via crop milk (Willemien et al., 2004).

Within the mammalian lineage two specialized structures to aid in this transfer have emerged, the placenta and the mammary gland. Strategies of immune transfer in mammals can also be considered ‘pre-natal,’ occurring in utero across the placenta, or ‘post-natal’ during lactation. A variety of strategies of passive immune transfer are seen among the mammalian lineages. For instance, a human baby acquires maternal circulating antibodies transplacentally and does not absorb any antibodies from the milk. Conversely, cows do not receive antibodies transplacentally and therefore are entirely reliant upon IgG absorption from the milk (Hurley and Theil, 2011; Murphy, 2012). Some species utilize a combination of transfer methods; goats for example acquire maternal antibodies both in utero and via the milk (Hurley and Theil, 2011). Lactation has played a crucial role in providing the developing offspring with necessary nutritional and immunological protection throughout the evolution of the mammalian lineages (Lefevre et al., 2007; Oftedal 2012).
**Lactation and Immune System Interaction**

The incorporation of immune protection into milk secretion requires the complex interplay of immune cell types and secretory molecules within the mammary gland. The highly vascularized nature of the alveoli allows for the association of maternally circulating immune components to be incorporated into the active mammary gland and milk production. The primary immunomodulatory components that are received passively are in the form of maternally derived antibodies. Immunoglobulin gamma (IgG) can be considered the workhorse immunoglobulin of passive immunity in eutherians. IgG is the primary antibody transferred across the placenta, and in animals that do not receive immunity \textit{in utero}, like the cow, it is the primary antibody transferred via lactation (Brambell, 1970; Larson, 1980). IgG is the main antibody isotype found in blood and extracellular fluids and serves as the primary antibody in secondary immune responses against infection (Murphy, 2012). IgG is transferred passively via the Neonatal Fc Receptor (FcRN) (Roopenian and Akilesh 2007).

Immunoglobulin A (IgA) is a specialized isotype crucial to mucosal immunity (Lycke and Bemark 2017). IgA is secreted in a dimeric form called secretory IgA (sIgA). The monomers of IgA are held together by the joining chain (J chain), like the other polymeric immunoglobulin, IgM (M for macro) (Murphy 2012). IgA is the primary antibody in mucosal secretions, but can also be detected in small amounts in the blood (Murphy, 2012). In animals that receive IgG transplacentally, IgA is the predominant antibody in the milk (Brambell, 1970, Larson, 1980). IgA is transported across epithelial barriers via the polymeric Ig receptor (pIgR). Unlike other transfer proteins that are recycled, a portion of the receptor is cleaved off and remains with the transported IgA dimer in the form of a secretory component (SC). The SC enhances the stability of IgA within the gut environment by protecting it from degradation (Lindh, 1975; Mostov, 1994).

The gut is one of the primary exposure routes to antigens and mucosal immunity provides a first line of defense against infection. A delicate balance needs to be maintained that
allows the immune system to react to potential pathogens, yet not remain in a heightened level of response to mutualistic colonizers. Persistent immune activation within the gut results in a sustained elevated inflammatory state, compromising the epithelial barrier of the intestine and allowing for bacterial translocation and subsequent infection of the host (Rogier et al., 2014). Maintaining this balance of selective tolerance and environmental restriction is primarily the function of IgA (Lee and Mazmanian, 2010).

Maternally derived antibodies transferred via the milk reflect antigenic stimulation of the mucosa-associated lymphoid tissues (MALT) and the specificity of the antibodies transferred are likely against infectious agents in the mother’s environment that the neonate is likely to encounter (Brandtzaeg, 2010, Murphy, 2012). The exact role of maternally derived IgA has yet to be fully established in neonates that do not absorb maternal secretory IgA into circulation, however the IgA may serve as a maternal mechanism of regulating the establishment and maintenance of the enteric microbiome (Pacheco et al., 2015).

Other Ig isotypes found in milk include IgM and Ig epsilon (IgE). IgM is polymeric, typically a pentamer, in mammals, a feature that serves to amplify immune recognition of first encountered antigens and will elicit other non-specific immune clearance mechanisms. IgE is best known in its relationship to human allergy although evolutionarily it appears to have a role in combating helminth infections (Bell, 1996). The role of IgM and IgE in passive immunity has not been fully investigated throughout the lineages.

Lymphocytes, both B and T cells, have been seen to infiltrate the eutherian mammarys during lactation. The function of lymphocytes within the mammarys has primarily focused on antibody producing B cells. Investigations into eutherian mammary T cell populations during active lactation are limited. Most studies focus on lymphocyte subtypes in milk secretions or during pathological states such as infection or cancer (Sharp et al., 2016; Stanton and Disis 2016). Among eutherians, mammary CD4+ and CD8+ αβ T cells as well as γδ T cells were detected, but variation in proportions of these
subtypes exist between species (Reardon et al., 1990; Ismail et al., 1996; Chabaudie et al., 1997). For example, in healthy, active goat mammary tissue, CD4⁺ T cells predominated; in contrast, in healthy, active cow mammary tissue CD8⁺ T cells predominated (Ismail et al., 1996; Yamaguchi et al., 1999). In general, mammary T cell numbers in eutherians tend to increase during gestation and decline throughout lactation into weaning and involution (Manning and Parmley, 1980; Salmon and Delouis, 1982; Reardon et al., 1990; Ismail et al., 1996; Chabaudie et al., 1997; Yamaguchi et al., 1999; Salmon et al., 2009).

The role of leukocytes within the milk is still under debate and concentrations of cell types vary amongst species (Hassiotou et al., 2013). It has been suggested that high concentrations of phagocytic leukocytes within the milk serve as non-specific immune protection of the developing neonate (Young et al., 1997). Other leukocytes, like eosinophils and macrophages, have been detected within developing mammary glands and have been speculated to play a fundamental role in mammary development however the mechanism remains unknown (reviewed in Reed and Schwertfeger, 2010).

Other non-specific innate immune compounds in milk include defensins and transferrins. Defensins have been analyzed in human and bovine milk primarily and serve as a source of antimicrobial peptides (AMPs) inhibiting the overgrowth of potentially pathogenic bacteria (Martin et al., 1995; Mohanty et al., 2015). Transferrins are iron-binding proteins that control the concentration of free iron within biological fluids. Milk-specific transferrins are referred to as lactoferrins (Baker et al., 2002). Lactoferrins exert bacteriostatic properties by sequestering free iron essential for bacterial growth (Lonnerdal, 2003). These compounds appear to serve a supplemental role in the establishment and maintenance of the developing neonatal enteric microbiome.

**Marsupial Acquisition of Passive Immunity**

A major distinction between eutherian and marsupial neonates is the state of their physical and immunological development at birth. Marsupials are born highly altricial without a functional adaptive immune system, in contrast most eutherians are born with a
functional albeit naïve adaptive immune system (Tyndale-Biscoe and Janssens, 1988; Old and Deane, 2000; Parra et al., 2009; Wang et al. 2012; Rechavi et al., 2015). This results in an increased reliance on maternal factors for immunological protection of neonates.

_M. eugenii_ is the only marsupial known to transfer IgG _in utero_, in which substantial quantities of IgG have been detected in the vascular tissue of the yolk sac placenta and fetal serum (Renfee 1973; Samples et al., 1986; Deane et al. 1990). IgG levels of the pouch young continue to rise after birth as well prior to the offsprings’ ability to produce endogenous IgG, suggesting postnatal uptake of maternal antibodies through the gut via the milk (Deane et al., 1990). IgA was not detected in fetal serum prior to suckling, suggesting the only transfer of IgA occurs across the mammary epithelium into the milk and gut of the neonate (Deane et al., 1990). No other marsupial species analyzed to date has displayed this strategy of maternal transfer _in utero_ and instead are entirely dependent upon the milk for acquisition of maternally derived immunity (Samples et al., 1986; Deane et al., 1990; Old and Deane, 2000; Belov et al., 2002; Edwards et al., 2012).

Along with changing nutritional composition, changes in immunological composition have been described in marsupial milk that serves as a mechanism of protecting the immune-incompetent offspring. Investigations into the immunological composition of marsupial milk have been ongoing within several species since the early 1970’s (Yadev and Eadie, 1973; Hindes and Mitzell, 1976; Deane and Cooper, 1984; Samples et al., 1986; Deane et al., 1990; Adamski and Demmer, 1999; 2000; Daly et al., 2007; Joss et al., 2009). These early investigations noted low concentrations of IgG within the milk and speculated total immunoglobulin levels in marsupial milk were lower than that of eutherian species (Deane and Cooper, 1984). This was based on generalizations within other mammalian lineages in which there is little to no IgG transferred transplacentally, but is the primary antibody transferred in the milk (Butler, 1974).

A biphasic pattern of increased IgG expression has been reported within the mammarys of _M. eugenii_ and _T. vulpecula_ (Adamski and Demmer 1999; 2000; Daly et al., 2007; Joss et al., 2009). The first period of increased immune transfer markers corresponds with
parturition, and the second increase occurs around the transition to late lactation, when
the offspring is transitioning to life outside the pouch. This period of transition has been
likened to a “second birth” as offspring are more morphologically, developmentally, and
immunologically mature (Russell 1982; Dean and Cooper 1988; Adamaski and Demmer
1999; 2000). Evacuation from the pouch would also represent an immunologically
vulnerable stage for the offspring, as the pouch has been known to provide a level of
protection from potential pathogens (Edwards et al. 2012).

This same biphasic pattern has been reported in the mammarys of the *M. eugenii* for
other immunoglobulin isotypes as well (Daly et al., 2007). All immunoglobulin isotypes
displayed a biphasic pattern of expression with up-regulation at parturition and again at
the transition to late lactation (Daly et al., 2007). IgM and IgE expression have
previously only been investigated in *M. eugenii* lactation. In both *M. eugenii* and *T.
* 
*vulpecula* IgA was the predominantly expressed immunoglobulin and remained at an
elevated level of expression throughout lactation relative to other immunoglobulins
(Adamski and Demmer, 2000; Daly et al., 2007; Joss et al., 2009). Both relevant
immunoglobulin transporters, pIgR and FcRN, displayed a similar biphasic expression
pattern throughout lactation suggesting highly coordinated gene expression at the
maternal level related to transfer of passive immunity (Adamski and Demmer, 1999;
Adamski et al., 2000; Daly et al. 2007).

A remarkable correlation between timing of expression of mammary secretions and the
developing neonate occurs within marsupials. Evidenced by increased levels of FcRN
expression in neonatal gut corresponding with increased IgG concentration in the milk at
the transition to late lactation in *T. vulpecula* (Western et al., 2003). The biphasic
expression pattern of immune genes and reported changes in immunological composition
of the milk, reported within marsupial species appears to occur at a time in which the
offspring are likely most vulnerable to potential pathogens: following parturition and
prior to evacuation of the pouch. To date it is unknown whether this pattern is conserved
across all marsupial species, including those of the Americas.
**Monodelphis domestica- Model Marsupial**

The gray short-tailed opossum, *Monodelphis domestica*, is a small marsupial from South America utilized for decades in captive bred laboratory settings (VandeBerg and Robinson, 1997). It has a well-sequenced and annotated reference genome, enhancing genetic studies in this species (Mikkelsen *et al*., 2007). *M. domestica* is a non-seasonal breeder, making them an ideal candidate for reproduction-related studies. Litter size can vary from 1-13 with fecundity related to a multitude of factors, but litter sizes ranging 8-12 are not uncommon (VandeBerg and Williams-Blangero, 2010; BDF observations). Females and males reach sexual maturity by 5 and 6 months of age, respectively. Females will enter estrous within 3-13 days following pairing with a male and subsequent exposure to male pheromones (Trupin and Fadem, 1982; Fadem, 1985; Fadem, 1987; Baggott *et al*., 1987; Kraus and Fadem, 1987; VandeBerg, 1990; Stoonerook, and Harder, 1992; Kuehl-Kovarik *et al*., 1995; VandeBerg and Williams-Blangero, 2010). Ovulation and fertilization occur approximately 24 hours post-mating (Mate *et al*., 1994). Marsupial embryos are encapsulated in a maternally derived shell coat which ruptures in late gestation and superficially implants into the uterine wall, limiting the ability of maternally-derived immunoglobulin transfer *in utero* (Moffett and Loke, 2006). Indeed previous research demonstrated that passive immunity from maternally derived sources is derived strictly from the milk and does not occur *in utero* in *M. domestica* (Samples *et al*., 1986). This is likely related to the limited invasiveness of the placenta.

Like other marsupials, *M. domestica* has a short gestation period, of only 13.5 days post-fertilization, resulting in the birth of highly altricial young (Samples *et al*., 1986, Mate *et al*., 1994, Kuehl-Kovarik *et al*., 1995). The developmental stage of the neonates is comparable to that of a day-13 rat embryo a day-10 mouse embryo or a day-40 human embryo (Kuehl-Kovarik *et al*., 1995; VandeBerg and Williams-Blangero, 2010). Following parturition *M. domestica* pups, which have been described as a “living tube”, will crawl onto their mother’s abdomen and attach to one of her 13 teats (Kuehl-Kovarik *et al*., 1995). The teat will swell to the size of their mouth, essentially sealing their mouths from the environment, and they will remain continuously attached for
approximately two weeks (Samples et al., 1986; Kuehl-Kovarik et al., 1995). During this period offspring will undergo a dramatic increase in size and morphological development. Teats without a pup will begin to regress and will not progress through subsequent phases of lactation and instead enter a quiescent stage (Hinds 1988; BDF observation unpublished).

*M. domestica* has one of the shortest lactation times relative to gestation among the marsupials. Eighty percent of opossum maternal time is invested in lactation compared to 93% in *M. eugenii*. Opossum lactation consists of only eight weeks as well, compared to 50 weeks in *M. eugenii* and 29 weeks in *T. vulpecula* (Nicholas et al., 1997; Kuy et al., 2007). *M. domestica* offspring are weaned in a laboratory setting after 56 days. In one study *M. domestica* lactating dams were allowed to remain co-housed with their offspring up to 75 days postpartum for the purposes of milk collection and characterization of nutritional components of milk, at which time no further milk production was noted (Crisp et al., 1989). It is unknown whether cessation of lactation was prompted from a lack of suckling stimulus by the offspring, genetic programming for cessation or inability for the dam to keep up with the energy demands of the offspring. This compressed lactation scheme may influence the lactation scheme in the opossum compared to previous reports in Australian marsupials.

The changing nutritional composition of milk throughout lactation in *M. domestica* is similar to that of other marsupials (Crisp et al., 1989; Green et al., 1991). However, *M. domestica* offspring behavior does not follow the same pouch emergence behaviors as described in *M. eugenii* as *M. domestica* lacks a *marsupium*, or pouch, making pouch emergence behavior an unreliable metric for the transition from early to late lactation.

*M. domestica*, like other marsupial species, is born in an immunologically incomplete state of development further highlighting the reliance on acquisition of passive immune protection (Samples et al., 1986; Parra et al., 2009; Wang et al., 2012). Lymphoid tissue in marsupials is undifferentiated at birth and does not contain defined cortical or medullary regions until a week or more after birth (Deane and Cooper, 1988). The timing
of appearance of functional lymphocytes within *M. domestica* neonates has been established.

Light chain expression, indicative of mature B cells, is not detected in *M. domestica* until postnatal day 7 (Wang *et al.* 2012). B cells undergoing isotype switch to production of IgG have not been detected prior to the fifth postnatal week. B cells that have undergone isotype switching to IgA production are not detected until the eighth postnatal week, suggesting an obligatory role of maternally derived IgA in regulating establishment and maintenance of the developing neonates enteric microbiota (Wang *et al.* 2012). Class switch to IgA has been reported at an earlier stage in some marsupial species, even prior to detection of IgG (Belov *et al.*, 2002). This may be representative of living condition and early pathogen exposure, as early IgA class switch was detected in wild-caught opossums in contrast to the controlled environment of laboratory rearing utilized with *M. domestica* studies.

T cell commitment occurs within the last 24 hours before birth in *M. domestica* and functional transcripts of T cell receptors (TCR) for conventional α, and β can be detected as early as postnatal day 1. However, functional TCRγ transcripts were not detected until day 8 (Parra *et al.*, 2009; Hansen and Miller 2017). This suggests that αβT-cells develop prior to γδT-cells *M. domestica* in contrast to Eutherian mammals (Parra *et al.*, 2009).

Like all jawed vertebrates, marsupials have both αβ and γδ T cells (reviewed in Hansen and Miller, 2015). In addition, the marsupials have a third T cell lineage expressing the TCR μ chain. TCRμ is a fifth TCR chain unique to marsupials and monotremes and the phenotype of TCRμ+ T lymphocytes remains unknown (Parra *et al.*, 2007; Parra *et al.*, 2012; Wang *et al.*, 2011). Functional TCRμ expression can be detected 2-3 weeks postnatal suggesting delayed development of TCRμ+ T lymphocytes (Parra *et al.*, 2009).

Although the ontogeny of immune development has been well established in this model, information on key systems such as lactation is still lacking. Investigations into potential correlations occurring within the mammarys would further enhance the model as well as our understanding of the evolution of lactation in mammals.
Aims of this dissertation

The aims of the research presented in this dissertation were centered on investigating mammary development and markers of passive immune transfer within the mammary glands throughout the course of lactation in the model marsupial *Monodelphis domestica*. A combination of histology, immunohistochemistry and quantitative PCR were used in these investigations. Results presented in this dissertation further enhance the model *M. domestica* as well as provide insight into the evolutionary role of the immune system in mammary development and passive immune transfer.

Chapter 2:
**Fehrenkamp, B.D.** and R.D. Miller (2018) **Histological maturation of the opossum mammary as it relates to immune cell infiltration and nutritional gene transcription** *Proceedings of the Royal Society B: Biology*

This chapter is currently in preparation to be submitted to the *Proceedings of the Royal Society B: Biology*. This chapter focuses on morphological restructuring in the mammary associated with pregnancy and lactation, including eosinophil presence within the developing mammary glands. Presence and abundance of nutritive-associated gene transcripts, including marsupial specific proteins, were quantified and correlated with mammary development.

Chapter 3:

This chapter was submitted for a special issue of *Reproduction, Fertility and Development*, Reproduction Down Under and is currently in press. This chapter focuses on presence and abundance of markers associated with passive immune transfer to offspring via the mammary. This manuscript presents evidence of coordinated
expression of FcRN within maternal mammary glands and the gut of developing offspring. Patterns of IgA and pIgR expression also coordinate with offspring development.

Chapter 4:

**Fehrenkamp, B.D.** and R.D. Miller (2018) *γδ T cells are the predominant T cell type in opossum mammary glands during lactation.* *Developmental and Comparative Immunology.*

This chapter in currently in preparation to be submitted to *Developmental and Comparative Immunology.* This chapter focuses on characterizing T cell presence within the mammary glands throughout lactation. Presence and abundance of transcripts encoding T cell subset markers were also quantified within the mammary glands throughout lactation. This manuscript presents evidence of the potential role of *γδ* T cells within the developing mammary glands.

Chapter 5: Summation Chapter
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Chapter 2

Histological maturation of the opossum mammary as it relates to immune cell infiltration and nutritional gene transcription

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Abstract

The mammary gland has evolved in species-specific ways to accommodate the developmental needs of offspring. This appears particularly true for marsupials. Marsupial milk content changes throughout lactation in ways that appear timed with neonatal ontogeny and behavior. *Monodelphis domestica*, the gray short-tailed South American opossum is a valuable model system and one in which there is still limited information on key systems like lactation. Here we investigate morphological restructuring within the mammary glands throughout lactation, including the presence of eosinophils in the tissue. Substantial remodeling of the mammary glands occurs throughout the first half of lactation. Eosinophils were present in early developing mammary tissue. Eosinophil presence correlates with increased transcript abundance of the chemokine IL-16. Opossum mammary glands appeared developmentally similar to active eutherian mammary glands in the later half of lactation. The presence and abundance of whey protein transcripts within the mammary glands were quantified as well. Whey acidic protein abundance peaked in the later half of lactation and remained elevated through weaning. Minimal transcripts for marsupial specific Early and Late Lactation Proteins (ELP/LLP) were detected in active lactation. Elevated LLP abundance was only detected prior to parturition. These results support a conserved role for eosinophils in mammary development across marsupial and eutherian lineages and describe key similarities and differences in whey protein transcript abundance across marsupial species.
Introduction

An innovation in synapsid evolution is the mammary gland that serves as a specialized structure for milk production that provisions developing neonates (Oftedal, 2012). There is growing evidence of the role of cells of the immune system in the development and function of mammarys. Comparative studies can provide insights into the origins of these roles. A significant group of mammals for such studies are the marsupials. Marsupials are a lineage of mammals that diverged from eutherians approximately 165 million years ago (Bininda-Emonds et al., 2007). Marsupials give birth to altricial young that complete developmental stages resembling eutherian fetal development, but outside of the womb, often in a pouch structure (Tyndale-Biscoe and Janssens 1988). Marsupials switch over from dependence on placental provisioning to dependence on milk earlier in their development than eutherians. (Tyndale-Biscoe and Renfree, 1987).

The eutherian mammary is an example of a reproductive tissue that undergoes substantial remodeling during pregnancy. This includes an increase in secondary and tertiary ductal branching, providing a network for the development of the alveoli where milk synthesis and storage occurs (Djonov et al., 2001; Macias and Hinck, 2012). Cells of the immune system, eosinophils in particular, participate in eutherian mammary development and remodeling (Reed and Schwertfeger, 2010). These eosinophils appear to be attracted to the site by the cytokine Interleukin (IL)-16 (Rand et al., 1991). By parturition eutherian mammary development is complete and there is little postnatal change in architecture (Djonov et al., 2001; Macias and Hinck, 2012).

To gain a greater understanding of the evolution of the development of mammary tissue we investigated mammary architecture in a model marsupial, the gray-short tailed opossum, *Monodelphis domestica*, This opossum is arguably one of the better marsupial model species, with a sequenced and well-annotated genome (Mikkelsen et al. 2007). The opossum has a short gestation and extended lactation period. Development of the immune system is initiated just prior to birth and mostly occurs postnatally (Parra et al. 2009; Wang et al. 2012; Hansen and Miller, 2017). Here we describe for the first time the
changes in mammary architecture throughout the lactation period with correlation to changes in immune cell composition and key nutritional gene transcript abundance.
Materials and Methods

**Animals and Tissue Collection**

*M. domestica* used were from a captive-bred research colony housed at the University of New Mexico Department of Biology Animal Research Facility. This study was approved under protocol numbers 16-200407-MC and 15-200334-B-MC from the University of New Mexico Institutional Animal Care and Use Committee. Animals were euthanized by inhaled isoflurane overdose until no evidence of breathing or heartbeat for one minute, followed by decapitation. For prenatal tissue, pregnancies were timed as described previously (Hansen et al., 2017).

Mammary tissue for RNA isolation was collected from at least three females at each time point. This included the last 24 hours of pregnancy (embryonic day (E) 13). In addition tissues were collected from post-partum (P) days 1, 2, 3, 5, 7, 10, 13, 16, 17, 20, 22, 26, 31, 32, 33, 36, 38, 44, 52. Post-weaning tissue was collected from mothers on P57 and P58, 24-48 hours after pups had been removed and housed separately at P56 (Supplementary Table 1). The number of pregnancies prior to the pregnancy when tissue was collected ranged from 0-6 with a median of 2. Tissues were preserved in RNALater buffer (Ambion) at 4 °C for 48 hours. The buffer was then removed and tissues were stored at -80 °C until extraction.

Mammary tissues from E3 and E13, as well as P3, 7, 10, 13, 17, 26, 33, 36, and 44 were also collected and preserved for histology following the methods of Old and Deane (2003). Tissues were preserved in 10% buffered formalin (Sigma Aldrich) at 4 °C for 24 - 48 hours and then washed repeatedly in 70% ethanol solutions to remove any residual formalin, before being dehydrated and embedded in paraffin wax. Embedded tissues were sectioned to 6 microns and mounted to Apex Superior Adhesive Glass slides (Leica).

**Histology**

For morphological examinations paraffin embedded mammary sections were Hematoxylin and Eosin (H&E) stained and preserved by cover slipping with DPX
Single field of view bright field microscopy was performed on an inverted Ti Nikon using NIS Elements imaging software (Nikon).

**RNA extraction and cDNA synthesis**

Whole RNA was extracted from mammary tissues using the Pure Link RNA mini kit and treated using the TURBO DNA-free Kit according to manufacturers’ recommended protocols (Ambion). 500 ng of RNA was used for cDNA synthesis by reverse transcriptase PCR (RT-PCR) using SuperScript III First Strand Synthesis kit (Invitrogen). To reduce bias generated during reverse transcription, reactions were constructed in triplicate and pooled.

**Quantifying Gene Transcripts**

Transcript abundance of specific genes was assessed by quantitative real-time PCR (qPCR) using Sso Advanced Universal SYBR Green Supermix (BioRad) according to manufacturer’s instructions for 20 µL reactions. qPCR was performed in triplicate on a BioRad CFX96. Primers were designed for the *M. domestica* genome according to manufacturer’s parameters for qPCR primers (Supplementary Table 2). Transcript abundance was normalized for each individual using the Vandesompele method of incorporating multiple reference gene expression levels (Vandesompele *et al.*, 2002). Reference genes used in this study were succinate dehydrogenase subunit A (*SDHA*) and actin-related protein 2 (*ACTR2*).

**Statistical analyses**

All statistical analyses were performed using default parameters of Prism 7 software (GraphPad). Normalized abundance was calculated per biological replicate per time point. Mean expression was calculated per biological set. Grubbs outlier analyses were performed across each biological set. Normalized expression of biological replicates including significant outliers is represented in Supplementary Figure 1. Inclusion of outliers did not significantly alter statistical analyses. Data, not including outliers, were pooled by week (Supplementary Table 1). Mean normalized abundance and standard error of the mean (SEM) were reported per gene target for each week.
Results

The opossum *M. domestica* is arguably among the better-established model species for studying the interplay of evolution, development, and genomics in marsupials. Nonetheless, gaps remain in basic descriptions of essential organ systems. Here we investigated morphological changes that opossum mammarys undergo throughout lactation and correlated these with the timing and abundance of specific nutritional gene transcripts. Mammary tissue from three biological replicates per time point across eleven time points spanning from gestation through the first seven of the eight weeks of lactation were examined for the presence of alveolar development and ductal branching (*Figures 1, 2, Supplementary Table 1*).

Prior to parturition opossum mammary gland development was minimal with few alveoli present. Alveoli are the regions where milk is being produced and stored. The tissue was predominately connective tissue and abdominal muscle (*Figure 1A, B*). Increased alveoli number was detected within the first week of lactation, however connective tissue still predominated and minimal signs of ducting were present (*Figure 1C, D*). By the second week of lactation early duct structures were evident. Alveoli increased both in number and size in week 2 of lactation compared to earlier time points (*Figure 1E, F*). By the third week of lactation alveoli continued to increase in size and secondary ducting was also evident (*Figure 1G*).

Cell density within a given field of view decreased throughout opossum lactation, as more of the mammary space was dedicated to milk storage (*Figure 1, 2*). The presence of highly defined alveoli and tertiary ductal branching was not evident until week 4 (*Figure 2A*). It was not until week 5 and after, in which the alveoli encompassed the majority of the fat pad and structured ducting was evident throughout the tissue (*Figure 2B - D*). Enlarged alveoli and decreased connective tissue were seen weeks 5 and beyond. Late lactation, weeks 5 and older, opossum mammary tissue appeared developmentally equivalent to active eutherian mammary tissue (*Figure 2B - D*). Histological remodeling of opossum mammary tissue early in lactation correlates developmentally to restructuring
that occurs during pregnancy in eutherians (Djonvi 2001; Macias and Hinck, 2012). An increased maturity in opossum mammary development

Figure 1: Morphological changes within opossum mammarys throughout early lactation.
corresponds with previously reported changes in nutritional composition and quantity of milk produced (Green et al. 1991).

Mammary development in eutherians has been associated with the presence of eosinophils (Reed and Schwertfeger, 2010). Eosinophils were evident in early, postnatal week 1, opossum mammary sections as well (Figure 3). In P3 mammary sections eosinophils were detected surrounding alveoli in early stages of development and were commonly found in the stroma surrounding developing alveoli. When mammary tissues from weeks 2 through 7 were examined eosinophils were not observed (not shown). IL-16 has been found to be chemotactic for eosinophils in other species (Rand et al., 1991).
When IL-16 transcript abundance in the opossum mammary fat pad was investigated using qPCR it was significantly elevated following parturition and decreased linearly through weaning (Figure 4), and correlated well with the presence of eosinophils (Figure 3).

**Figure 3: Eosinophil presence within early lactating opossum mammary fat pad.**
40X magnification of P3, week 1, mammary tissue counterstained in hematoxylin. Arrows indicate eosinophils. Scale in lower right = 10 µm.

**Figure 4: IL-16 is elevated early in lactation.**
IL-16 abundance is highest in the first week postpartum and decreases linearly throughout weaning (slope = -0.2399, y-intercept = 2.195, $r^2 = 0.848$, and $p = 0.0004$). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. Dotted horizontal line denotes normalized expression = 1. Pooling as described in supplementary table 1.
Two milk proteins unique to marsupials are Early Lactation Protein (ELP), and Late Lactation Protein (LLP) (Nicholas et al., 1987; Joss et al., 2007). Their functions remain unknown. Nonetheless, they have been used in studies of Australasian marsupials to define changes in milk composition (Kuy et al., 2007; Joss et al., 2007). The M. domestica genome contains a single gene for ELP and a single gene for the LLP-B paralogue.

To establish if the presence of ELP and LLP correlates with morphological change in the opossum mammarys, the abundance of transcripts encoded by these genes was determined. No ELP transcripts were detected in mammarys prior to birth (Figure 5). Low levels of transcripts were first detected in late in postpartum week 1 with minor peaks in abundance at weeks 2 and 4, however these increases were not statistically different from zero (Figure 5, Supplementary Figure 1A). LLP transcripts were detected in elevated abundance in the last 24 hours prior to parturition and declined to low levels throughout postnatal lactation (Figure 5, Supplementary Figure 1C). Other than the peak of LLP transcripts late in pregnancy, neither ELP nor LLP were detected in great abundance at any time point across active lactation in the opossum mammarys.
Figure 5: Early Lactation Protein (ELP) and Late Lactation Protein (LLP) transcript abundance within the opossum mammary glands. Transcript abundance of ELP (Blue circles) and LLP (Red squares) by week. Annotations as in Figure 4.

Another protein used in Australasian marsupials to describe changes in nutritional composition is Whey acidic protein (WAP) (Nicholas et al., 2001; Demmer et al. 2001; Kuy et al., 2007). WAP transcripts were not detected in opossum mammary glands until the second week of lactation (P10) and followed a bell-shaped pattern from week 2 through 8 with peak abundance at week 5 (Figure 6, Supplementary Figure 1A).

Figure 6: Whey Acidic Protein (WAP) transcript abundance within the opossum mammary glands throughout lactation. Transcript abundance of WAP by week. Annotations as in Figure 4.
Discussion

There are three living lineages of mammals, differing significantly in their methods of provisioning offspring during development. The two live-bearing lineages, the marsupials and the eutherians, are noteworthy in the relative contributions the placenta versus the mammary glands provide the fetus and newborn. This has led many investigators to relate postnatal development in marsupials to “fetal” development in the eutherians (Tyndale-Biscoe and Janssens 1988). In many ways, this comparison holds up. Many of the developmental stages that occur in the prenatal eutherian do not take place in the marsupial until following birth. For example, major systems like respiratory, excretory, and even adaptive immune components develop postnatally in the opossum (Yadev et al., 1972; Basden et al., 1997; Buaboocha and Gemmell, 1997; Parra et al., 2009; Wang et al., 2012).

One might expect that differences in the pre- and postnatal development in marsupial versus eutherian young would be recapitulated in the mammary glands supporting this development. The results of this study address this question and lead to a number of conclusions regarding what has been conserved across mammals, what is conserved among marsupials, and what is not conserved.

The overall structure of the opossum mammary, throughout development during lactation, is very similar to that of eutherians. The distinct difference is the timing relative to birth and the onset of milk production. In eutherians remodeling of mammary tissue occurs during pregnancy in preparation for milk production (Djonov et al., 2001; Macias and Hinck, 2012). By late pregnancy, secondary and tertiary ductal branching is present in eutherian mammary glands and alveoli encompass the majority of the fat pad, however there is little milk production per se. (Djonov et al., 2001). In contrast, in the opossum there is little alveolar development, nor ducts, in maternal mammary glands at parturition in spite of the immediate onset of milk production. Ductal branching was not present in opossum mammary glands until week 3 of active lactation and alveoli did not encompass the majority of the tissue until week 5 and throughout the rest of lactation.
(figure 1, 2). In other words, the developmental state of the mammaries at birth in eutherians is not achieved until postnatal week 5 in the opossum.

We found that the presence of eosinophils in mammary tissue during early remodeling is conserved between eutherians and marsupials. Eosinophils were present in opossum mammary tissue in the first postnatal week, but not at later time points, correlating well both with when remodeling of the tissue is occurring as well as when IL-16 transcripts were most abundant. This is consistent with IL-16’s role in eosinophil chemotaxis previously reported (Rand et al., 1991). Eosinophils are believed to be fundamental in developing eutherian mammary tissue however their exact role remains unknown (Reed and Schwertfeger, 2010).

The time line of mammary remodeling in the opossum is compressed when compared to other marsupial species. Enlarged alveoli and less dense stroma were not seen in tammar wallaby until 37 weeks into active lactation compared to 5 weeks in the opossum (Findlay, 1982; Nicholas 1988). This is not surprising given the differences in developmental growth rate and time to weaning between opossums and the larger macropods. Opossums are typically weaned at 56 days compared to 350 days in the tammar wallaby (VandeBerg and Robins 1997; Nicholas et al. 1997).

The opossum mammarys reached their peak development in appearance at postnatal week 5. At week 5 the offspring are not independent, however are covered in fur, have opened their eyes, and are beginning to seek solid food (VandeBerg and Williams, 2010; BDF observations). They have a more developed adaptive immune system and appear to be becoming less dependent on maternal immunity (Wang et al 2012; Parra et al., 2009). Specifically there is a decrease in transcription of FcRN, the transporter for IgG, in neonatal intestine at this time suggesting a transition from dependence on maternal IgG when the offspring begins producing their own (Fehrenkamp et al., 2018).
Figure 7: Morphological remodeling correlates with offspring development and nutritional composition.

Morphological development of the mammary correlates with previously described changes in composition and increased quantity of milk production (Green et al., 2001). Histological photos are representative images from gestation E3, P3 (week 1), P16 (week 3), P32 (week 5), and P44 (week 7) in order of appearance from left to right. Offspring developmental images are from the last 24 hours of pregnancy (E13), P3 (week 1), P13 (week 2), P20 (week 3), P24 (4), P32 (week 5) and P44 (week 7).

The whey-associated protein with the greatest conservation of expression among marsupials appears to be WAP. WAP has been described as a ‘mid-late’ lactation protein in Australian marsupials (Simpson and Nicholas 2000; Demmer et al., 2001; Kuy et al., 2007; Joss et al., 2009). In the opossum, WAP transcripts were detected starting week 2 with greatest abundance week 5. It is noteworthy that this peak abundance correlates with maximal mammary maturity. Based on transcript abundance WAP would likely be the predominant protein within the milk from early-middle lactation through weaning (Figure 5, 7, not shown). The transcript abundance results shown here also correlate
well with previous reports of general protein abundance in opossum milk (Green et al. 1991). WAP transcripts were detected throughout lactation well into weaning in the opossum. This is in contrast to tammar wallaby and brushtail possum where WAP transcripts decline to undetectable by weaning (Demmer et al., 2001; Kuy et al., 2007; Joss et al., 2009).

Figure 8: Schematic comparison of phase specific gene expression across lactation in three marsupial
Stage specific whey protein transcript abundance is represented in opossum, *M. domestica* (top panel), tammar wallaby, *M. eugenii* (middle panel), and Australian common brushtail possum, *T. vulpecula* (bottom panel). Relative timing of detected elevated expression of ELP (blue bar), LLP (red), and WAP (green) are represented. Expression in *M. eugenii* and *T. vulpecula* were adapted from (Trott et al., 2003). Research in *M. eugenii* and *T. vulpecula* did not distinguish between LLP-A and LLP-B homolog expression. Only the LLP-B homolog exists in the opossum and is reported here.

The opossum genome contains a single gene copy encoding what has been described in other marsupials as LLP. In wallabies, there are two LLP genes, A and B, and the
A marsupial opossum gene appears orthologous to the LLP-B gene (Joss et al., 2009). When combined with the results from two Australian marsupial species, we find three different patterns of LLP expression. In the opossum LLP transcripts were only detected early, prior to birth. In the tammar wallaby it has only been found later in lactation, hence the origins of the name: Late Lactation Protein. Lastly, in the brushtail possum, it is found both early and late (Figure 8) (Demmer et al., 2001).

Based on the pattern of expression in tammar wallaby, LLP has been speculated to play a role in the transition to an herbivorous diet (Nicholas et al. 1987). While this is certainly a possibility in a herbivorous species like the wallaby, it does not appear to be the case in the opossum. Likewise it is unknown if there is a biological significance to the wallaby having two, paralogous copies of the LLP gene, nor what role LLP might play surrounding parturition in opossum and brushtail possum.

Among the whey-associated proteins, opossums differ the most from other marsupials in the presence of ELP. In tammar wallabies and brushtail possums this protein is found early in lactation and declines at the time of the appearance of LLP. Opossums have an ELP orthologue, however, transcript abundance was low throughout lactation. The role of ELP remains unknown, making it difficult to speculate on the species differences.

Based on analyses of changing milk composition, mammary transcriptome, and offspring behavior investigators have subdivided marsupial lactation into phases. They have even speculated the universality of these phases across marsupials (Nicholas, 1988). While the opossum does appear to have both qualitative and quantitative changes throughout lactation it is not clear that the phases described in Australian marsupials would translate. This could be a reflection of differences in natural histories, or could represent 69 million years of evolutionary history separating Australasian and American marsupials (Nilsson et al. 2004).
Conclusions

Marsupial mammary development occurs at different stages relative to milk production compared to eutherians. In which mammary development that occurs during pregnancy in eutherians occurs during early milk production in marsupials. This development occurs more rapidly in the opossum and morphological restructuring of the mammary correlates with previously reported changes in nutritional composition of the milk as well as offspring development. Eosinophil infiltration and increased transcript abundance of IL-16 is also evident in early developing opossum mammary and is suggestive of a conserved role for leukocytes in developing mammary tissue across mammalian lineages.
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Author contributions
BF carried out the tissue collection, molecular lab work, histological work, data analysis, participated in the design of the study and drafted the manuscript; RM conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

Conflicts of interest
The authors declare no conflicts of interests.
References


**Supplementary Material**

**Supplementary Table 1**: Summary of time points of mammary collection, number of biological replicates and associated method of preservation.

<table>
<thead>
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<th>Tissues Used in Histological Examinations</th>
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**Supplementary Table 2:** Primer information. Primer sequences, annealing temperature and efficiency is reported for each gene target as well as associated Ensembl gene code.

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<th>Primer location</th>
<th>Annealing Temperature (°C)</th>
<th>Amplicon length (cDNA)</th>
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Supplementary Figure 1: Transcript abundance by day, ELP (A), LLP (B), and WAP (C). Significant outliers were identified for LLP only. Outliers are included here.
Chapter 3

Opossum milk IgG is from maternal circulation and timing of transfer correlates with neonatal immune development.

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Abstract

Marsupials, with short gestation times, have more complex and changing patterns of milk composition compared to eutherians. Maternal immunoglobulins (Ig) that confer immunity on offspring are among the components that change during marsupial lactation. Here the abundances of mammary transcripts encoding Ig heavy chains and their corresponding transporters were quantified in the laboratory opossum, *Monodelphis domestica*. IgA transcripts are the most abundant in opossum mammary and, with IgM, increased in abundance linearly from birth to weaning. Likewise, the Fc receptor for IgA, the poly-Ig receptor, also increased in abundance throughout lactation. There were few transcripts for IgG or IgE within the opossum mammarys. This is in contrast to that reported for Australian marsupial species. Transcripts for FcRN, the Fc receptor that transports IgG, were detected throughout lactation, and opossum milk is known to contain IgG. Therefore, milk IgG is likely to be taken from maternal circulation, rather than local production. There is a parallel increase in FcRN in the newborn gut that declines around the time when neonates have matured to the point they can make their own IgG. These results are consistent with a transfer of maternal Ig that is coordinated with the development of the neonatal immune system.
Introduction

Milk provides newborn mammals with nutritional as well as immunological value. There is variation, however, across the mammalian lineages on the relative contributions of each of these sets of factors. The contrast is particularly striking when comparing lactation in eutherians to that of marsupials, two mammalian lineages separated by 165 million years (Bininda-Emonds et al. 2007). Immediately following birth, eutherian mammarys produce a unique type of secretion called colostrum. Colostrum is rich in nutrients and antibodies (reviewed in Hurley and Theil 2011). Following the colostral phase, eutherian species produce milk of a relatively consistent composition throughout lactation until weaning (reviewed in Jenness and Sloan 1970; Skibiel et al. 2013). In contrast, the equivalent of the colostral phase is debatable in marsupials and the composition of milk, both nutritionally as well as immunologically, changes during lactation (reviewed in Edwards et al. 2012). These changes appear to relate to the developmental states of the marsupial neonates and likely are an evolutionary adaptation to the short intra-uterine gestation times and limited placentation.

A general pattern of changing nutritional composition within the milk has been described in numerous marsupial species. Early in lactation milk is dilute and contains low levels of proteins and fats with high concentrations of carbohydrates. Later in lactation milk composition transitions to a higher concentration of proteins and fats with little carbohydrates (reviewed in Green and Merchant 1988). The changing nutritional composition as well as offspring behavior has led to the assignment of phases to marsupial lactation (reviewed in Tyndale-Biscoe and Renfree, 1987).

Marsupial lactation has been most extensively studied in the tammar wallaby (*Macropus eugenii*). Tammar wallabies have two periods of increased expression of immunoglobulins (Ig) and Ig transporters within the mammarys, first following birth and again at the time of transition to weaning (Daly et al. 2007; Joss et al. 2009). Similar patterns have been described in the common brushtail possum (*Trichosurus vulpecula*)
although the phases are less defined within common brushtail possums (Adamski and Demmer 1999; Adamski and Demmer 2000; Adamski et al. 2000).

The South American opossum, *Monodelphis domestica*, has been the primary model for American marsupials, which diverged from their Australian cousins at least 60 million years ago (Bininda-Emonds et al. 2007). Investigations into macromolecule composition of opossum milk found similar changes throughout lactation and a universal strategy of lactation among the marsupial lineage has been suggested (Crisp et al. 1989; Green et al. 1991). The existence of distinct phases of lactation have not been clearly defined within the opossum. *M. domestica* lacks a pouch and as such pup behavior does not follow the same pouch emergence behaviors as described in tammar wallabies.

Opossum offspring, like other marsupials, are born at an underdeveloped state compared to eutherian neonates (Old and Deane 2000; Deane and Cooper 1988). Opossums are born developmentally equivalent to that of a day-10 mouse embryo, a day-13 rat embryo, or a day-40 human embryo (La Via et al., 1963; Kuehl-Kovarik et al. 1995; VandeBerg and Robinson, 1997). Neonatal opossums lack many of the cells of the adaptive immune system during the first postnatal week and there is no evidence of *in utero* transfer of maternal Ig (Samples et al. 1986; Parra et al. 2009; Wang et al. 2012). Newborn opossums, therefore, appear entirely reliant on milk for acquiring passive immunity.

Opossum, a species with a sequenced and annotated genome, has only four of the five Ig heavy chain isotypes, IgM, IgG, IgA and IgE, described in mammals (Mikkelsen et al. 2007; Wang et al. 2009). Here we investigated the abundance of transcripts encoding each of these four Ig isotypes and their corresponding Ig transporters, the neonatal Fc receptor (FcRN) and poly-Ig receptor (pIgR), within mammary tissue across the course of opossum lactation.
Materials and Methods

Animal Use and Tissue Collection
Opossums used were from a captive-bred research colony housed at the University of New Mexico Department of Biology Animal Research Facility. This study was approved under protocol numbers 16-200407-MC and 15-200334-B-MC from the University of New Mexico Institutional Animal Care and Use Committee.

Opossum mammary tissues were collected from timed pregnancies as described previously (Hansen et al., 2017). Females used in this study were observed daily and the morning pups were first sighted was counted as postnatal/postpartum day (P) 1. Mammary tissues were collected from at least three females at each time point. Time points collected for this study were the last 24 hours of pregnancy (embryonic day 14, E14) and post-partum (P) days 1, 2, 3, 5, 7, 10, 13, 16, 17, 20, 22, 26, 31, 32, 33, 36, 38, 44, and 52. Mammary tissues were also collected from animals 24-48 hours after pups had been weaned on day 56, (post weaning). Intestinal tissue from at least 3 individuals per time point was collected from pups from postnatal day (P) 5, 7, 10, 16, 22, 32, 36, and 45. Adult opossum intestinal tissues were also collected to serve as controls.

Animals were euthanized by inhaled isoflurane overdose until no evidence of breathing or heartbeat for one minute, followed by decapitation. Tissues were preserved in RNALater buffer (Invitrogen, Carlsbad, CA, USA) at 4°C for 48 hours. The buffer was then removed and tissues were stored at -80°C until extraction.

RNA Extraction, and cDNA synthesis
RNA was extracted using phenol based extraction methods utilizing Pure Link RNA mini kit (Invitrogen, Carlsbad, CA, USA). 10 µg of whole RNA was treated with DNase using the TURBO DNA-free Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturers’ recommended protocols. 500 ng of DNA cleaned RNA was used to make cDNA pools by reverse transcriptase PCR (RT-PCR) using SuperScript III First Strand Synthesis kit.
(Invitrogen, Carlsbad, CA, USA). To reduce bias generated during reverse transcription, cDNA synthesis reactions were constructed in triplicate and pooled.

**Quantifying Specific Gene Transcripts**

Abundance of specific gene transcripts was assessed by Quantitative real-time PCR (qPCR) using the Sso Advanced Universal SYBR Green Supermix (BioRad, Hercules, CA, USA) according to manufacturer’s guidelines for 20µL reactions. qPCR reactions were performed in triplicate on a BioRad CFX96. Primers used in qPCR were designed for the opossum genome according to manufacturer’s parameters for qPCR primers. All primer pairs were designed to span introns in the genomic DNA to distinguish amplification of cDNA. Primer pairs designed to amplify Ig heavy chain encoding transcripts were based on constant (C) region gene sequence. Primer sequences, properties and other pertinent qPCR parameters are reported in Supplementary Table 1. A negative control (no template) reaction was included for each primer pair.

Mammary transcript abundance per targeted gene was normalized for each individual by the Vandesompele method of incorporating multiple reference gene expression levels (Vandesompele et al., 2002). Reference genes used in this study were succinate dehydrogenase subunit A (SDHA) and actin-related protein 2 (ACTR2). These genes were highly expressed at all time points with little variance. They also had M values < 1 as defined in Vandesompele and colleagues (2002). Transcript abundance of FcRN within the neonatal and adult intestines was normalized for each individual by the Pfaffl method utilizing ACTR2 (Pfaffl 2001).

**Statistical Analyses**

All statistical analyses were completed utilizing the default parameters of Prism 7 software (Graphpad, La Jolla, CA, USA). Grubbs’s outlier analyses were performed to examine for significant outliers within each biological set. All outliers identified per target gene are reported in the Supplementary Figure legends. Mean normalized transcript abundance for biological set was calculated with and without outliers. Biological set average or weekly average is reported per target including standard error of the mean.
(SEM). Analysis of variance (ANOVA) as well as Pearson’s correlation analyses were calculated for both means and compared for statistical variance. Inclusion of outliers did not significantly alter results, albeit statistical power was decreased with inclusion. Normalized expression of all outliers is represented for each gene target (shown in Supplementary Figures), however was omitted for reported weekly mean expression.
**Results**

To investigate presence and abundance of transcripts encoding Igs and Ig transporters, mammary tissue was examined from at least three individuals from a total of 21 time points. These time points spanned from the last 24 hours of pregnancy (E14) to 24-48 hours after removal of pups at weaning.

IgA heavy chain transcripts were amplified in qPCR using primers specific for the Ig Cα constant region gene. IgA transcript abundance was found to increase throughout the course of opossum lactation with E14 (week 0) and week 1 being significantly lower than P52 (week 8) ($p \leq 0.01$, $p \leq 0.05$ figure 1A, Supplementary Figure 1). There was a slight dip in transcript abundance during week 5 that is significantly lower than elevated abundance seen in week 8 ($p \leq 0.01$). The greatest individual variation was observed around the time of weaning.
Figure 1: Normalized mammary transcript abundance of IgA (A) and pIgR (B). Linear regression values for IgA were $p = 0.0014$, $r^2 = 0.7395$, slope $= 0.2945$. Values were normalized to reference genes, ACTR2 and SDHA. Dotted lines indicate normalized expression $= 1$. Data were pooled by week where week 0 is E14; week 1 is P1, 2, 3, 5, and 7; week 2 is P10 and 13; week 3 is P16, 17, and 20; week 4 is P22 and 26; week 5 is P31, 32, and 33; week 6 is P36 is 38; Week 7 is P44; and week 8 is P52. The final point is post weaning. *$p \leq 0.05$, **$p \leq 0.01$.

Given the steady increase of IgA transcripts throughout lactation the mammary transcript abundance of the IgA transporter, pIgR, was also investigated. Transcript abundance of pIgR was found to follow a similar pattern as that of IgA (figure 1B). A linear increase in pIgR transcript abundance is evident throughout lactation, however statistical significance was not reached ($p = 0.0758$). This correlation is stronger when examining individual time points but still fails to reach statistical significance ($p = 0.0530$, Supplementary Figure 2). The largest variation in pIgR transcript abundance was found
after the removal of offspring at weaning. Transcript abundance of pIgR and IgA appears to be highly coordinated throughout lactation except for surrounding parturition (E14 and P1) when pIgR transcripts are significantly more abundant than that for IgA (p ≤ 0.01, figure 2).

![Image](image_url)

**Figure 2:** Abundance of IgA and pIgR transcripts appears to be highly coordinated throughout lactation. IgA (gray line) and pIgR (black line) transcript abundance reported by day. Annotations as in Figure 1.

Similar to IgA, IgG transcript abundance was quantified by qPCR using primers specific for the Cγ constant region gene. IgG transcripts were the least abundant immunoglobulin isotype within the mammarys, with few transcripts detected at any time point (figure 3A, Supplementary Figure 3). A slight increase in abundance is evident in week 1 and week 6 however these increases are not statistically significant and represent only a few transcripts.
Given little evidence of resident IgG production in the opossum mammary glands, it is likely that milk IgG is transferred directly from maternal circulation in this species. To investigate this further, FcRN transcript abundance in mammary glands was examined. A prominent peak was found surrounding week 4 of lactation (figure 3B, Supplementary Figure 4). A significant increase in transcript abundance was detected between week 1 and week 4 ($p < 0.05$), followed by a decrease in week 5. Postnatal week 5 was previously found to be the time when neonatal opossums begin producing endogenous IgG (Wang et al., 2012). Neonates use FcRN to transfer milk IgG into newborn circulation from the gut lumen (Roopenian et al., 2007). To investigate if there is coordinated timing of FcRN in the newborn gut with that of the mammary glands; transcript abundance for FcRN was examined within intestinal tissue of neonates as well. FcRN
transcripts were most abundant during the first three weeks of postnatal life and declined between weeks 3 and 5, and remained at baseline until weaning (figure 4).

![Figure 4: Normalized transcript abundance of FcRN in neonatal opossum gut. Individual expression was normalized by reference gene ACTR2. Annotations as in Figure 1.](image)

A positive linear increase in IgM transcripts was found in the mammary glands across the course of lactation (p = 0.0002, figure 5, Supplementary Figure 5). The largest variation in IgM transcript abundance occurred around the time of weaning. The joining chain (J-chain) utilized in the formation of polymeric immunoglobulins also displays a linear increase in mean transcript abundance throughout lactation similar to that of IgM and IgA (not shown).
Figure 5: Normalized mammary transcript abundance of IgM. Linear regression values for IgM were $p = 0.0002$, $r^2 = 0.8373$, slope = 0.08963. Annotations and data pooling for weeks are as in Figure 1. Transcripts of IgE are in low abundance throughout lactation (not shown). Minimally elevated transcript abundance is present during week 1 and week 4 of lactation, however these differences are not statistically significant and represent relatively few transcripts.
Discussion

Some Australian species of marsupials have been found to have complex, changing patterns of mammary Ig transcripts; patterns that appear to correlate with the developmental stages of their neonates (Trott \textit{et al.} 2003). Comparable analyses, however, have yet to be reported for opossum, or any American marsupial. In this study we investigated the abundance of mammary transcripts for all four antibody heavy chain isotypes found in opossum, as well the corresponding Ig transporters, FcRN and pIgR.

IgG does not appear to be transcribed within the opossum mammarys during lactation at any time point. Furthermore, endogenous neonatal IgG synthesis is not evident in opossum neonates until postnatal week 5 (Wang \textit{et al.} 2012). Nonetheless, previous research has detected circulating IgG within offspring immediately following the initiation of suckling (Samples \textit{et al.} 1986; Wild \textit{et al.} 1994). Collectively these observations are consistent with IgG in newborn opossums being transferred from maternal circulation rather than \textit{de novo} synthesis in the mammarys. This hypothesis is supported by the detection of mammary FcRN transcripts prior to birth. The greatest abundance of mammary FcRN transcripts occurs during week 4 (figure 6), just prior to the detection of endogenous IgG transcripts in offspring at week 5 (Wang \textit{et al.} 2012). Similarly, the peak abundance of neonatal gut FcRN transcripts was during the first three postnatal weeks and declines at a time approaching when they begin to produce their own IgG. This coordinated decline in the transfer of maternal IgG with neonatal immune development would allow the young opossums to begin to develop their own immune repertoires and memory.
Figure 6: Immune related transcripts within the mammary and neonatal gut correspond with offspring immune development. Comparison of results presented in Figures 1, 3, 4, and 5 to developmental milestones in the neonates. Adapted from Wang et al 2012.

In contrast to the results presented here for opossum, the Australian tammar wallaby has IgG transcripts in the mammary tissues, consistent with local production (Daly et al. 2007). FcRN is fairly consistently transcribed throughout lactation in Australian species (Adamski et al. 2000; Daly et al. 2007). These results have lead investigators to conclude previously that the IgG in tammar wallaby and common brushtail possum milk is endogenous to the mammarys.

IgA is the immunoglobulin isotype predominantly transcribed within the mammarys of all marsupial species studied, including now the opossum. A biphasic, two-peak pattern of increased IgA expression has been described in both tammar wallabies and common brushtail possums (Adamski and Demmer 1999; Daly et al. 2007). It has been proposed the first peak surrounding parturition times correlates with the early establishment of the
enteric microbiome. The second peak, occurring around the transition to late lactation, correlates with the offspring beginning to supplement their diet with solid food in preparation for weaning (Daly et al. 2007). Opossums lack this biphasic pattern. Rather IgA transcripts were found throughout opossum lactation and increased in abundance over time. Opossums do not make IgA until 8 weeks of age, just prior to weaning (Wang et al. 2012). Continuous production of mammary IgA in the opossum may be necessary due to the delayed ability of the pups to produce this isotype endogenously.

It is interesting to speculate on the difference between the source of milk IgG and IgA in the opossum. The benefit of extracting IgG from maternal circulation rather than local production may be to provide the young with a spectrum of antibody, broadly specific for pathogens for which the mother has developed immunity. In contrast, IgA is specialized for mucosal sites and, for example, in humans antibody-producing B cells first activated in the gut transit to the mammaries of lactating women (Goldblum et al. 1975; reviewed in Brandtzaeg 2010). This provides the offspring with milk IgA specific for gut microbes. Whether such B cell trafficking occurs in opossums is not yet known, however our results would be consistent with this possibility.

IgM transcripts in opossum mammaries increased linearly throughout lactation, much like IgA. There is little evidence of IgE production in opossum mammaries. These are both also in contrast to the tammar wallaby that displays a biphasic pattern of IgM and IgE production (Daly et al. 2007; Joss et al. 2009).

The differences between the American opossum, and Australian tammar wallabies and common brushtail possums, may be due to speciation and differences in life-history traits such as body size, the presence of a pouch, diets, relative duration of lactation, etc. In particular, the pouchless nature of *M. domestica* may result in a greater dependence on maternal antibodies. However, another confounding influence might be husbandry. The *M. domestica* used in this study were indoor, laboratory bred from a well-established colony and free of any known pathogens. The Australian studies likely used animals from more natural, wild conditions, with complex immune histories and potential
pathogen exposure. How species differences versus immune history may influence the results are not known. Nonetheless, based on the results presented here, there does not appear to be a universal lactation scheme amongst the marsupial lineage.
Acknowledgements

The authors would like to acknowledge the contributions of Ali Salehpoor, for his assistance with mammary tissue extractions. We would like to thank Dr. Victoria L. Hansen for the use of her drawing in figure 6. We would like to thank the staff of the UNM Biology Animal Research Facility for their assistance with husbandry and care of the *M. domestica* colony.

This research was funded in part by a National Science Foundation award No. IOS-13531232. Research reported in this publication was supported in part by the National Institute of General Medical Sciences of the National Institutes of Health under award number P30 GM110907. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflicts of interest

The authors declare no conflicts of interests.
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Vandeberg, J.L., and Robinson, E.S. (1997). The laboratory opossum (Monodelphis domestica) in laboratory research. *ILAR Jour.* 38; 1; 4-12. [https://doi.org/10.1093/ilar.38.1.4](https://doi.org/10.1093/ilar.38.1.4)


Supplementary Material

Supplementary Table 1: Gene specific primer characteristics utilized in qPCR.

Amplification cycling parameters consisted of an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and GSP annealing temperature (varied see table) for 30 s, a terminating step of 95°C for 5 s terminating at 65°C for 31 s. A final melt curve was constructed by 60 cycles of 65°C for 5 s increasing +0.05°C/cycle with a ramp of 0.05°C/cycle. Singularity of product size was also examined per plate by melt curve analyses. A sample from the serial dilution was run on a 2% agarose gel and stained with RedGel Nucleic Acid Stain and viewed under UV light to confirm that a band of the correct size was amplified. Efficiency of the amplification was determined for each primer pair using serial 10 fold dilutions of pooled cDNA. All calculations were performed using BioRad CFX 3.1 (BioRad). Reference gene appropriateness was evaluated using calculated M values and target stability scores across all samples. Gene studies were constructed using BioRad software and an interplate calibrator was utilized across all plates. Several attempts were made to locate efficient IgG primers that spanned an intron. The product of the primer pair reported for IgG was analyzed for singularity of product by the presence of a single band in PCR, melt curve analyses, as well as cloning and sequencing of product. Sequenced products had 100% identity and alignment with target sequence.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Ensembl gene ID</th>
<th>Target use</th>
<th>Primer Sequences</th>
<th>Primer location</th>
<th>Annealing Temperature (°C)</th>
<th>Amplicon length (cDNA)</th>
<th>Primer efficiency</th>
<th>Calibration curve slope</th>
<th>Calibration curve y-intercept</th>
<th>Calibration curve r²</th>
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<td>ACTR2</td>
<td>Actin Related Protein 4</td>
<td>ENSMODG00000003364</td>
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<td>F: TGATCAACGTGGAAGGAGTG  R: ATCCTCCAGAAAGACGATG</td>
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<td>FCRN</td>
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<td>ENSMODT00000017389</td>
<td>Target</td>
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<td>3.441</td>
<td>44.083</td>
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<td>ENSMODG000000016627</td>
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<td>Exon 2</td>
<td>65</td>
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<td>0.993</td>
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<td>ENSMODG000000016626</td>
<td>Target</td>
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<td>107.3%</td>
<td>-3.150</td>
<td>43.118</td>
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Supplementary Figure 1: Normalized mammary transcript abundance of IgA. IgA transcript abundance by day. Linear regression values were $p = 0.0001$, $r^2 = 0.5441$, slope = 0.03646. One individual at P1, P3, P17, P20, and P33 was identified as a significant outlier among the replicates. Statistical analyses were performed with and without inclusion of these individuals. Inclusion did not significantly alter the overall significance of the analyses. Outlier expression is represented for each time point but has not been included in the mean expression among the biological set shown here. * $p \leq 0.01$. 

![Graph showing normalized expression of IgA transcript abundance by day.](image-url)
Supplementary Figure 2: Normalized mammary transcript abundance of pIgR. pIgR transcript abundance by day. Linear regression analyses failed to reach significance (p = 0.0530). One replicate at P36 was identified as a statistically significant outlier. Statistical analyses were performed with and without inclusion of outliers as in Supplementary Figure 1. *p ≤ 0.05, **p ≤ 0.01
Supplementary Figure 3: Normalized mammary transcript abundance of IgG. IgG transcript abundance by day. Significant outliers were identified at 6 time points, E14, P3, P13, P17, P31, and P44. The outlier at P3 was identified as a significant ‘over-expressor’ for IgG as well as IgM and IgE. This individual may have been experiencing an immune response within the mammary not related to passive immune transfer at collection that was unknown. Statistical analyses were performed with and without inclusion of outliers as in Supplementary Figure 1.
Supplementary Figure 4: Normalized mammary transcript abundance of FcRN. FcRN transcript abundance by day. *p ≤ 0.05 **p ≤ 0.01. Significant outliers were detected at E14, P3, P13, P17, P31, and P34. Statistical analyses were performed with and without inclusion of outliers as in Supplementary Figure 1.
Supplementary Figure 5: Normalized mammary transcript abundance of IgM. IgM transcript abundance by day. Linear regression values were $p = 0.0053$, $r^2 = 0.3433$, slope = 0.008895. Significant outliers were detected at P3, P13 and P44. Statistical analyses were performed with and without inclusion of outliers as in Supplementary Figure 1.
Chapter 4

\( \gamma \delta \) T cells are the predominant T cell type in opossum mammary glands during lactation.

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Keywords:
mammary, lactation, marsupial, T cell, \( \gamma \delta \) T cell, eosinophil, IL-16
Abstract

Milk provides mammalian neonates with nutritional support and passive immunity. This is particularly true in marsupials where young are born highly altricial and lacking many components of a fully functional adaptive immune system. Here we investigated the T cell populations in the mammary glands of a lactating marsupial, the gray short-tailed opossum *Monodelphis domestica*. Immunohistochemistry confirmed the presence of T cells within the opossum mammary glands throughout lactation. Results of quantifying transcript abundance for lymphocyte markers are consistent with γδ T cells being the most common T cell type within lactating mammas. Numbers of γδ T cells appear to peak early during the first postnatal week, and then decline throughout lactation until weaning. In contrast, numbers of αβ T cells and γμ T cells appear to be low to non-existent in the lactating mammary glands. The results support an ancient and conserved role of immune cells in the evolution and function of mammalian mammary tissue.
Introduction

A hallmark of mammalian biology is the dependence on milk for immunological and nutritional support during postnatal development. Metatherian mammals, or marsupials, are noteworthy for having a complex scheme of milk production; the nutritional and immunological content of milk changes during the course of lactation to support the progressive maturational stages of the young (Daly et al., 2007; Joss et al., 2009; Fehrenkamp et al., 2018). Much of the work on lymphocytes associated with marsupial lactation has focused on those in the milk itself (Cockson and McNeice, 1980; Young et al., 1997; Young and Deane, 2001). These studies were limited to morphological identification without further classification of lymphocyte types. Less is known regarding lymphocyte subsets within the marsupial mammary tissue throughout lactation.

Investigations into eutherian mammary T cell populations during active lactation are limited as well. Most studies focus on lymphocyte subtypes in milk secretions or during pathological states such as infection or cancer (Sharp et al., 2016; Stanton and Disis, 2016;). Among eutherians, mammary CD4+ and CD8+ αβ T cells as well as γδ T cells were detected, but variation in proportions of these subtypes exist between species (Reardon et al., 1990; Ismail et al., 1996; Chabaudie et al., 1997). For example, in healthy, active goat mammary tissue, CD4+ T cells predominated; in contrast, in healthy, active cow mammary tissue CD8+ T cells predominated (Ismail et al., 1996; Yamaguchi et al., 1999). In general, mammary T cell numbers in eutherians tend to increase during gestation and decline throughout lactation into weaning and involution (Manning and Parmley, 1980; Salmon and Delouis 1982; Reardon et al., 1990; Ismail et al., 1996; Chabaudie et al., 1997; Yamaguchi et al., 1999; Salmon et al., 2009).

Like all jawed vertebrates, marsupials have both αβ and γδ T cells (reviewed in Hansen and Miller, 2015). In addition, the marsupials have a third T cell lineage expressing the T cell receptor (TCR) μ chain. TCRμ is a fifth TCR chain unique to marsupials and monotremes and the phenotype of TCRμ+ T lymphocytes remains unknown (Parra et al, 2007; Wang et al., 2011; Parra et al., 2012). Here we investigated both the presence of T cells in the opossum mammary during lactation as well as the lineages of T cells present using transcript profiles.
Materials and methods

Animal use and tissue collection
Opossums used were from a captive-bred colony housed at the University of New Mexico Department of Biology Animal Research Facility. This study was approved under protocol numbers 16-200407-MC and 15-200334-B-MC from the University of New Mexico Institutional Animal Care and Use Committee.

For immunohistochemistry and histology, mammary tissues from at least three replicates from post-partum (P) days 3, 17, 32, and 44 were collected. For RNA isolation, at least three replicates per time point were collected from the last 24 hours of pregnancy (embryonic (E) day 14) and P1, 2, 3, 5, 7, 10, 13, 16, 17, 20, 22, 26, 31, 32, 33, 36, 38, 44, and 52. Mammary tissues were also collected from animals 24-48 hours after pups had been removed on day 56 (post weaning) (Supplementary Table 1). Opossum mammary tissues were collected from timed pregnancies as previously described (Hansen et al., 2017). Animals were euthanized by inhaled isoflurane overdose until no evidence of breathing or heartbeat for one minute, followed by decapitation.

Immunohistochemistry and microscopy
Tissues were preserved in 10% buffered formalin at 4 °C for 24-48 hours, washed repeatedly in 70% ethanol to remove any residual formalin before being dehydrated and embedded in paraffin wax consistent with previously published protocols (Old and Deane, 2003). Embedded tissues were sectioned to 6 microns and mounted on Apex superior adhesive slides (Leica Biosystems).

Formalin fixed paraffin embedded sections were de-paraffinized in xylene and rehydrated through graded alcohol solutions to PBS as previously described (Old and Deane, 2001). Antigen retrieval was completed by microwaving submerged sections in pre-warmed Sodium Citrate Buffer (pH = 6) for 20 minutes and allowing to cool to room temperature for at least 20 minutes. Peroxidase and biotin blocking were completed prior to overnight incubation at 4 °C with rabbit anti-human CD3ε polyclonal antibody (DAKO, A045229-2), a pan T cell marker proven to be cross reactive in marsupial species (Kreiss et al., 2009; Hansen and Miller, 2017). Sections were incubated with a polyclonal biotinylated goat anti-rabbit secondary antibody (DAKO, E0432) at
room temperature for two hours. Avidin binding (Vecastain, Vector Laboratories) and oxidation via DAB reagent (Abcam) resulted in a brown staining of positive lymphocytes. Sections were counterstained in hematoxylin (Thermo scientific) before dehydrating in graded ethanol solutions and coverslipped with DPX (Sigma Aldrich). Isotype, negative, and positive controls were included in optimization of immunohistochemistry conditions. All antibodies were diluted with 0.1% fish gelatin (Sigma Aldrich) 1X PBS solution in 1:500 dilutions. All incubations were performed in a humidified chamber and thorough washings in PBS followed all incubations and blocking procedures. Stained sections were imaged for single field of view on an Eclipse Nikon Ti inverted fluorescence microscope utilizing Nikon ARS (Nikon) software.

**RNA extraction and cDNA synthesis**
Mammary tissues were preserved in RNALater buffer (Ambion) at 4 °C for 48 hours. The buffer was then removed and tissues were stored at -80 °C until extraction. RNA was extracted from tissues using phenol based extraction methods utilizing Pure Link RNA mini kit (Ambion). 10 µg of whole RNA was cleaned of residual DNA using TURBO DNA-free Kit (Ambion). 500 ng of DNA-cleaned RNA was used to create cDNA pools by reverse transcriptase PCR using SuperScript III First Strand Synthesis (Invitrogen). cDNA synthesis reactions were constructed in triplicate and pooled to reduce bias generated during reverse transcription.

**Quantifying specific gene transcripts**
Transcript abundance of specific genes was assessed by quantitative real time PCR (qPCR) using Sso Advanced Universal SYBR Green Supermix (BioRad) according to manufacturer’s instructions for 20 µL reactions. qPCR reactions were performed in triplicate on a BioRad CFX96. Primers were designed based on opossum genome sequence according to qPCR manufacturer’s parameters (Supplementary Table 2).

Transcript abundances per gene were normalized for each individual by the Vandesompele method of incorporating multiple reference gene expression levels (Vandesompele et al., 2002). Reference genes used in this study were succinate dehydrogenase subunit A (SDHA) and actin-related protein 2 (ACTR2).
**Statistical analyses and graphical representation**

All statistical analyses were performed using default parameters of Prism 7 software (GraphPad). Normalized expression data were calculated per biological replicate per time point. Mean expression was then calculated per biological set. Grubbs outlier analyses were performed across each biological set. Normalized expression of biological replicates including significant outliers is represented in **Supplementary Figure 1**. Inclusion of outliers did not significantly alter statistical analyses. Data, not including outliers, were pooled by week (**Supplementary Table 1**). Mean normalized expression and standard error of the mean (SEM) are reported per gene target for each week.
Results

To investigate the presence of T cells within opossum mammary glands, immunohistochemistry was performed using an anti-CD3ε polyclonal antibody. Mammary sections from each of three individuals with pups at ages P3, 17, 32 and 44 were examined. The presence of mammary tissue CD3ε+ T cells was evident at all time points (Figure 1). They were located in sites of more mature alveoli. In addition to being within the mammary tissue, CD3ε+ T cells were also detected within mammary blood vessels, as well as in the alveolar milk space (Figure 2).

To phenotype and quantify the mammary T cells, the abundance of transcripts encoding various T cell specific markers was used as a proxy for the cell types present. Mammary tissues from at least three individuals per time point for 21 time points were used (Supplementary Table 1). These spanned from the last 24 hours of pregnancy (E14) through 24-48 hours following the removal of offspring at P56. TCR chain identity was determined from amplification using primers specific for the constant region of each chain.

Transcripts encoding the TCRα, β, γ, δ, and µ chains were quantified first. Abundances of TCRα and β chain transcripts were low at all time points (Figure 3). TCRα chain transcript abundance remained consistent throughout lactation with minor increases surrounding birth and weaning. TCRβ chain transcripts were partially elevated in week one, however this was due to increased abundance at P5 alone and no other time point (Figure 3, Supplementary Figure 1B). Normalized abundance of either chain transcripts was not significantly different at any time point (Figure 3). Abundance of CD4 and CD8 transcripts were also investigated (Figure 4). These T cell marker transcripts remained low throughout lactation.
Figure 1: CD3E$^+$ T lymphocytes are present within the mammary throughout lactation. Opossum mammary sections from P3 (A), P17 (B), P32 (C), and P44 (D) were examined for the presence of CD3E$^+$ T lymphocytes. Only lymphocytes with clear membrane staining and identifiable nucleus are indicated by arrow. CD3E$^+$ T lymphocytes were present at every time point investigated. Imaged at 40X magnification. Scale bar in lower right = 10$\mu$m.
Figure 2: CD3E⁺ T lymphocyte within the alveolar space in the mammaries.
P3 opossum mammary section at 40X magnification. CD3E⁺ T lymphocyte indicated by arrow. Scale bar in lower right = 10\(\mu\)m.

Figure 3: Normalized transcript abundance of TCR\(\alpha\) and \(\beta\) chains is minimal yet consistently detected.
TRAC (Circles) and TRBC (Squares) abundance appears coordinated throughout lactation. Dotted line denotes normalized expression = 1. For some points the error bar is shorter than the height of the symbol.
Figure 4: CD4 and CD8α transcript abundance is low yet consistently detected. CD8α (Circles) and CD4 (Squares) transcript abundance throughout lactation. Annotations as in figure 3.

The most abundant TCR transcripts were those encoding TCRγ chains (Figure 5). In marsupials, TCRγ can pair with either TCRδ or TCRμ, therefore transcripts were quantified for all three chains for comparison (Figure 5). The pattern of TCRγ transcript abundance was most closely overlapping with that of TCRδ. Indeed, there was a coordinated peak in abundance of both TCRγ and TCRδ transcripts following parturition that decreased linearly throughout the duration of lactation. In contrast, transcripts for the TCRμ chain were detected at only low levels, similar to patterns of TCRα and TCRβ. Minor increases in TCRμ transcript abundance were seen during weeks one and four, however these were not significant (Figure 5). These results are consistent with the TCRγ transcripts being primarily, if not entirely, from γδ T cells, and not γμ T cells.
Figure 5: TCR\(\gamma\) and TCR\(\delta\) chain transcripts are highly abundant early in lactation.

TCR\(\gamma\) (Squares) and TCR\(\delta\) (Triangles) abundance patterns appear most similar. TCR\(\gamma\) (thick line) and TCR\(\delta\) (thin line) decrease linearly from week 1 through weaning (slope = -0.1665, -0.1527, y-intercept = 1.748, 1.597, \(r^2 = 0.6731, 0.8776\), and \(p = 0.0067\) and 0.0002 respectively. TCR\(\mu\) (Circles) abundance is significantly lower than TCR\(\gamma\) during week 1 and 2. * \(p \leq 0.05\), ** \(p \leq 0.01\), *** \(p \leq 0.001\), **** \(p \leq 0.0001\). Annotations as in figure 3.

Discussion

The evolutionary innovation that defines mammals is the mammary gland. The mammary glands provide mammalian neonates with nourishment, as well as immune components that regulate the establishment of their microbiome and protect against infectious diseases (Goldman et al., 1998; Goldsmith et al., 2015). Cells of the immune system play a role in both the transmission of maternal immunity to offspring, and fundamentally contribute to the development and remodeling of mammary tissue (Reed and Schwertfeger 2010; Ofstedal, 2012).

Marsupials diverged from eutherians at least 165 million years ago and provide a unique comparison for mammary studies (Bininda-Emonds et al. 2007). Marsupials are noteworthy for having a relatively complex and dynamic lactation scheme (Daly et al., 2007, Joss et al., 2009; Fehrenkamp et al., 2018). The stage of mammary development at the onset of lactogenesis
differs greatly between the marsupial and eutherian lineages. In eutherian mammaries, development is complete by the end of gestation prior to the onset of lactation (Djonov et al., 2001). In contrast, in marsupials, milk production begins before the mammaries have completed their development. Substantial morphological restructuring continues to occur throughout early lactation (Hinds, 1988).

We have shown here that γδ T cells predominate as the mammary T cell type during lactation in opossums. The γδ T cells are most abundant in the first five weeks postpartum, corresponding to a time when the mammaries are undergoing their greatest remodeling (Hinds, 1988; Figure 5, not shown). It is noteworthy that the stage when γδ T cells are found in the opossum mammary most resembles the early developmental stages in eutherians when γδ T cells are also present. In eutherians this stage is during late gestation, whereas in marsupials it is during the early postnatal period when young are suckling (Reardon et al., 1990; Yamaguchi et al., 1999). These observations are consistent with a conserved role of γδ T cells within the developing mammaries in both marsupials and eutherians.

Recently, we characterized the B cell populations within opossum mammaries throughout lactation. We found there were primarily IgA producing cells. We hypothesized that IgA producing B cells were likely activated in the gut and had trafficked to the mammaries (Fehrenkamp et al., 2018). If the B cells were activated first in the gut as speculated there would be less need for T cell help in the mammaries. The low prevalence of CD4+ T cells within opossum mammaries shown here would be consistent with that hypothesis. In addition there are few if any IgG producing B cells within the mammaries and milk IgG is thought to be transferred from maternal circulation. This would also be consistent with a lack of need for CD4+ T cells in the opossum mammary.

Mammaries are a uniquely mammalian system whose origins can be illuminated through comparative approaches. Complex systems generally involve adaptation of preexisting systems to perform novel tasks. Components of the immune system, in particular, have been coopted into non-immune roles multiple times during evolution. For example, recent evidence supports a role of the immune system in the evolution of placentation and parturition in mammals (Hansen et
al., 2016; Griffith et al., 2017). Mammary glands are a complex tissue that likely evolved from cutaneous secretory glands (Oftedal, 2012). Our results using a marsupial, when compared to previous studies in eutherians, supports a role for γδ T cells in the evolution of mammary glands from these secretory glands.
Acknowledgements
The authors would like to acknowledge the contributions of Ali Salehpoor, for his assistance with mammary tissue extractions. We would like to thank the staff of the UNM Biology Animal Research Facility for their assistance with husbandry and care of the *M. domestica* colony.

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Conflicts of interest
The authors declare no conflicts of interests.
References


Supplementary Material

Supplementary Table 1: Time points of collection pooled by week including number of biological replicates per time point.

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</tr>
<tr>
<td>Post weaning</td>
<td>24-48 hours after removal of offspring at P56 (3)</td>
</tr>
</tbody>
</table>
Supplementary Table 2: Quantitative real-time PCR gene specific primer information.

Reactions were performed in triplicate on a BioRad CFX96. Cycling conditions were 95°C for 2:00 followed by 40 cycles of 95°C for 0:05 and 30 seconds at annealing temperature reported per target completed with 95°C for 5 seconds followed by a melting curve 65°C to 95°C in 0.5°C increments. Melt curve analyses assisted in identification of one product per reaction. Whenever possible primers were designed to span an intron. Amplification products were cloned and Sanger sequenced to confirm a singular product.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Ensembl gene ID/GenBank Accession</th>
<th>Target use</th>
<th>Primer Sequence</th>
<th>Primer location</th>
<th>Annealing Temperature (°C)</th>
<th>Amplicon length (cDNA)</th>
<th>Primer efficiency</th>
<th>Calibration curve slope</th>
<th>Calibration curve y-intercept</th>
<th>Calibration curve r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTR2</td>
<td>Actin-Related Protein 2 Homolog</td>
<td>ENSMODG000000013604</td>
<td>Reference</td>
<td>F: TGATCAACGTAACAGGAAGA</td>
<td>Exon 1</td>
<td>63</td>
<td>111</td>
<td>101.4%</td>
<td>-3.290</td>
<td>42.963</td>
<td>0.997</td>
</tr>
<tr>
<td>CD4</td>
<td>CD4</td>
<td>ENSMODG000000027590</td>
<td>Target</td>
<td>F: CTTTCGAGGAGAAGGAGA</td>
<td>Exon 4</td>
<td>63</td>
<td>131</td>
<td>100.8%</td>
<td>-3.488</td>
<td>38.434</td>
<td>0.983</td>
</tr>
<tr>
<td>CD8A</td>
<td>T-cell surface glycoprotein A</td>
<td>ENSMODG000000009515</td>
<td>Target</td>
<td>F: TGATTCGCTCTCGCTC</td>
<td>Exon 1</td>
<td>62</td>
<td>148</td>
<td>102.8%</td>
<td>-3.253</td>
<td>35.146</td>
<td>0.992</td>
</tr>
<tr>
<td>SDHA</td>
<td>Succinate dehydrogenase</td>
<td>ENSMODG000000006624</td>
<td>Reference</td>
<td>F: AAGAGGCTGTGGTCCTGAA</td>
<td>Exon 2</td>
<td>63</td>
<td>130</td>
<td>107.3%</td>
<td>-3.159</td>
<td>41.118</td>
<td>0.993</td>
</tr>
<tr>
<td>TRAC</td>
<td>Constant Region TCR α-chain</td>
<td>ENSMODG000000003530</td>
<td>Target</td>
<td>F: CTTGGCAGGATGACCAAG</td>
<td>Exon 1</td>
<td>64</td>
<td>195</td>
<td>96.9%</td>
<td>-3.399</td>
<td>42.948</td>
<td>0.976</td>
</tr>
<tr>
<td>TRBC</td>
<td>Constant Region TCR β-chain</td>
<td>GenBank Accession</td>
<td>Target</td>
<td>F: CTGGGCTGGGCTGGTGATG</td>
<td>Exon 1</td>
<td>64</td>
<td>10</td>
<td>98.2%</td>
<td>-3.582</td>
<td>45.807</td>
<td>0.991</td>
</tr>
<tr>
<td>TRDC</td>
<td>Constant Region TCR γ-chain</td>
<td>ENSMODG000000027573</td>
<td>Target</td>
<td>F: GAACAGTGGCTGGTGATG</td>
<td>Exon 2</td>
<td>62</td>
<td>94</td>
<td>95.1%</td>
<td>-3.444</td>
<td>41.268</td>
<td>0.996</td>
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<tr>
<td>TRGC</td>
<td>Constant Region TCR γ-chain</td>
<td>NCBI Gene ID:108059484</td>
<td>Target</td>
<td>F: GAACAGTGGCTGGTGATG</td>
<td>Exon 2</td>
<td>62</td>
<td>129</td>
<td>90%</td>
<td>-3.473</td>
<td>39.723</td>
<td>0.996</td>
</tr>
<tr>
<td>TRMC</td>
<td>Constant Region TCR γ-chain</td>
<td>GenBank Accession</td>
<td>Target</td>
<td>F: CGCGAAGGACCGAGAAGA</td>
<td>Exon 3</td>
<td>63</td>
<td>136</td>
<td>104.4%</td>
<td>-3.212</td>
<td>37.680</td>
<td>0.995</td>
</tr>
</tbody>
</table>
Supplementary Figure 1: Normalized mammary transcript abundance.

Transcript abundance by day. Significant outliers were identified for each gene target. Five individuals were identified as over-expressers for several gene targets. It is possible these outliers were experiencing an immune response within the mammary not detected in physical examination of tissue during dissection. A) TCRα, B) TCRβ, C) TCRγ, D) TCRδ, E) TCRµ, F) CD4, G) CD8α,
Chapter 5

Summation and Concluding Remarks

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Dissertation Summary and Concluding Remarks

The mammary gland is believed to have evolved from apocrine-like glands for the primary function of milk production (Oftedal 2012). All mammalian neonates are highly dependent on milk for continued nutritional and immunological provisioning postnatally. The majority of what is known regarding mammary development and the roles of lactation has been concerning eutherians, with limited investigations in other lineages. Comparative approaches are crucial to our understanding of the evolution of the mammary gland. Marsupials occupy an important phylogenetic position, being viviparous with the use of a complex lactation strategy. Marsupials are known for giving birth to highly altricial immunologically immature offspring that complete most of their development outside of the womb (Tyndale-Biscoe and Janssens 1988). This has resulted in a dynamic lactation scheme to further support the developmental needs of the neonates.

The majority of what was known about marsupial lactation, prior to this research, was regarding the Australian tammar wallaby, *Macropus eugenii*, with limited investigations in other Australian species. Investigations in American marsupials, which diverged from Australasian species approximately 69 million years ago, were limited beyond basic biochemical descriptions of milk composition (Crisp *et al.*, 1989a; 1989b; Green *et al.*, 1991; Nilsson *et al.* 2004). Comprehensive analyses into American marsupial lactation allow for comparative analyses between marsupial species as well as enhance our current understandings of the evolution of mammaries and lactation. The opossum, *Monodelphis domestica*, is arguably among the better-established model species for studying the interplay of evolution, development, and genomics in marsupials. The research presented in this dissertation describes for the first time comprehensive investigations into mammary development in a marsupial from pregnancy throughout lactation and investigates the roles of the immune system in opossum lactation.

Mammary development in marsupials is similar to that of eutherians however the timing of this development relative to the onset of lactation is significantly different across the lineages. In eutherians mammary development is complete prior to the end of pregnancy and the onset of lactation (Djonov *et al.*, 2001). In contrast, marsupial mammaries appear developmentally
similar to active eutherian mammaries at 5 weeks post-parturition, well beyond the onset of milk production (Chapter 2 Figure 2). We found that the presence of eosinophils during early mammary remodeling to be consistent across marsupial and eutherian species (Chapter 2 Figure 3). Eosinophils were present early in lactating opossum mammaries and correlated with elevated abundance of the chemokine IL-16 (Chapter 2 Figure 4). This supports an ancient and conserved role of eosinophils within the developing mammaries. What that role is remains unknown.

Examinations of whey protein transcript abundance within the opossum mammaries suggested a conserved pattern of appearance of whey-acidic protein (WAP) in marsupial milk, as a mid-late lactation protein. Patterns of usage of marsupial specific Early and Late Lactation proteins (ELP and LLP) were not consistent across marsupial species. Notably, LLP transcripts were only detected in elevated abundance in opossum mammaries prior to birth with limited abundance throughout active lactation. It is unknown the role LLP may be playing surrounding birth. ELP transcript abundance was minimal in the opossum mammaries at any time point and does not appear to play a major role in lactation in this species. The universal adoption of a marsupial specific lactation scheme may not be evident across marsupial species (Chapter 2 Figure 8).

To investigate passive immune transfer occurring via the mammaries in the opossum the presence and abundance patterns of immunoglobulin isotypes and relevant transporters were quantified in the mammaries throughout lactation. IgG transcripts were not detected within the mammaries at any time point. However the transporter for IgG, FcRN, was detected in the mammaries with peak expression week 4 (Chapter 3 Figure 3). I suggested that maternal IgG previously detected within suckling neonates, was transferred from maternal circulation via FcRN rather than local transcription. A coordinated pattern of FcRN abundance was evident in the mammaries and developing neonatal intestine, suggesting a decline in IgG transfer and acquisition correlating with offspring ability to produce IgG (Chapter 3, Figure 4,6) (Wang et al., 2012).

IgA abundance increased linearly throughout lactation and abundance patterns were similar to the abundance of the poly Ig receptor throughout active lactation (Chapter 3, Figure 1,2).
Transfer of maternal IgA throughout lactation is consistent with regulation of the neonatal microbiome until such time the offspring is capable of producing their own IgA near weaning. We also characterized the abundance patterns of the remaining opossum immunoglobulin isotypes, IgM and IgE. IgM abundance followed a similar pattern of increased abundance throughout lactation to IgA, however relative abundance was lower for IgM than IgA. IgE did not appear to play a major role in passive immunity in the opossum. This is in contrast to IgE detection within the mammaryes of the wallaby, however this may be related to the specific pathogen-free environment of the opossum. Differing patterns of immunoglobulin usage across marsupial species is consistent with adoption of variations in strategy across the lineage.

Consistent with our hypothesis that IgA producing B cells were activated in the gut and then trafficked to the mammaryes and IgG being transferred from maternal circulation rather than local production, minimal abundance of CD4 transcripts were detected in the opossum mammaryes throughout lactation (Chapter 4 Figure 4). T cells were evident within the mammaryes throughout lactation. However, γδ T cells were the predominant T cell subtype identified (Chapter 4 Figure 5). Interestingly, peak abundance of T cell receptor γ and δ transcripts within the mammaryes correlated with when the mammaryes were undergoing the greatest amount of remodeling. This is also consistent with reports of developing eutherian mammaryes and supports an ancient and conserved role for γδ T cells in developing mammary glands.

The results presented in this dissertation further enhance the marsupial model *M. domestica*. Ontogeny of immune development had been well-established previously within the model yet key information on mammary development and passive immune transfer via the mammaryes had yet to be explored. Here I show a dynamic relationship between immunoglobulin abundance patterns in opossum lactation and developing neonates. Results presented here also support an ancient and conserved role for integration of cells of the immune system including innate (eosinophils) and adaptive lineages (γδ T cells) in developing mammary glands.
References


