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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 mammaries. 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 mammaries 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 mammaries, 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 0.01, p 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 0.01). The greatest individual variation was observed around the time of weaning.

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).

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 mammaries, 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 mammaries, it is likely that milk IgG is transferred directly from maternal circulation in this species. To investigate this further, FcRN transcript abundance in mammaries 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 mammaries; 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).

A positive linear increase in IgM transcripts was found in the mammaries 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).

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 *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 mammaries during lactation at any time point. Furthermore, endogenous neonatal IgG synthesis is not evident in opossum neonates until postnatal week 5 (Wang *et al.* 2012). Nonetheless, previous research has detected circulating IgG within offspring immediately following the initiation of suckling (Samples *et al.* 1986; Wild *et al.* 1994). Collectively these observations are consistent with

IgG in newborn opossums being transferred from maternal circulation rather than *de novo* synthesis in the mammaries. 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 *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.

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 led investigators to conclude previously that the IgG in tammar wallaby and common brushtail possum milk is endogenous to the mammaries.

IgA is the immunoglobulin isotype predominantly transcribed within the mammaries 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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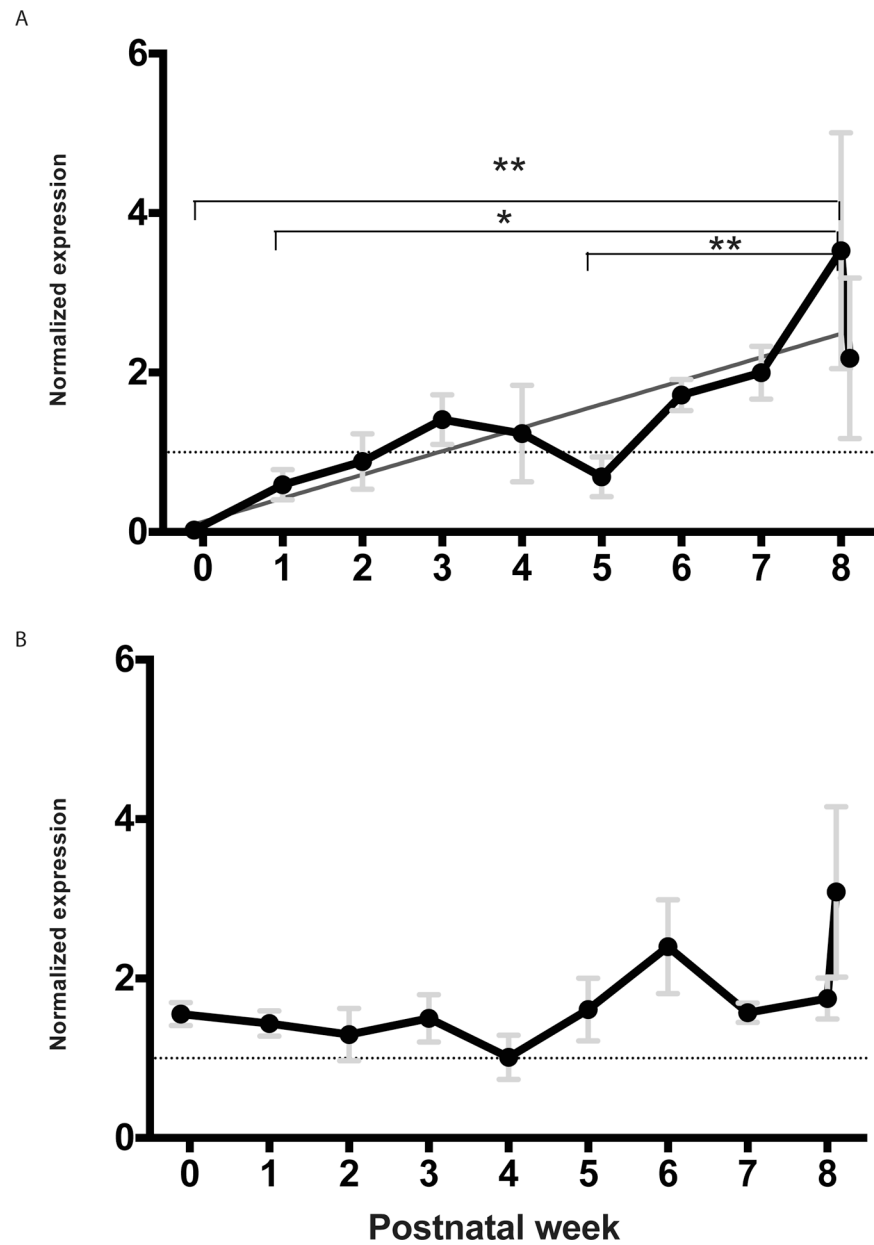
A

B

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 ≤ 0.05, **p ≤ 0.01.
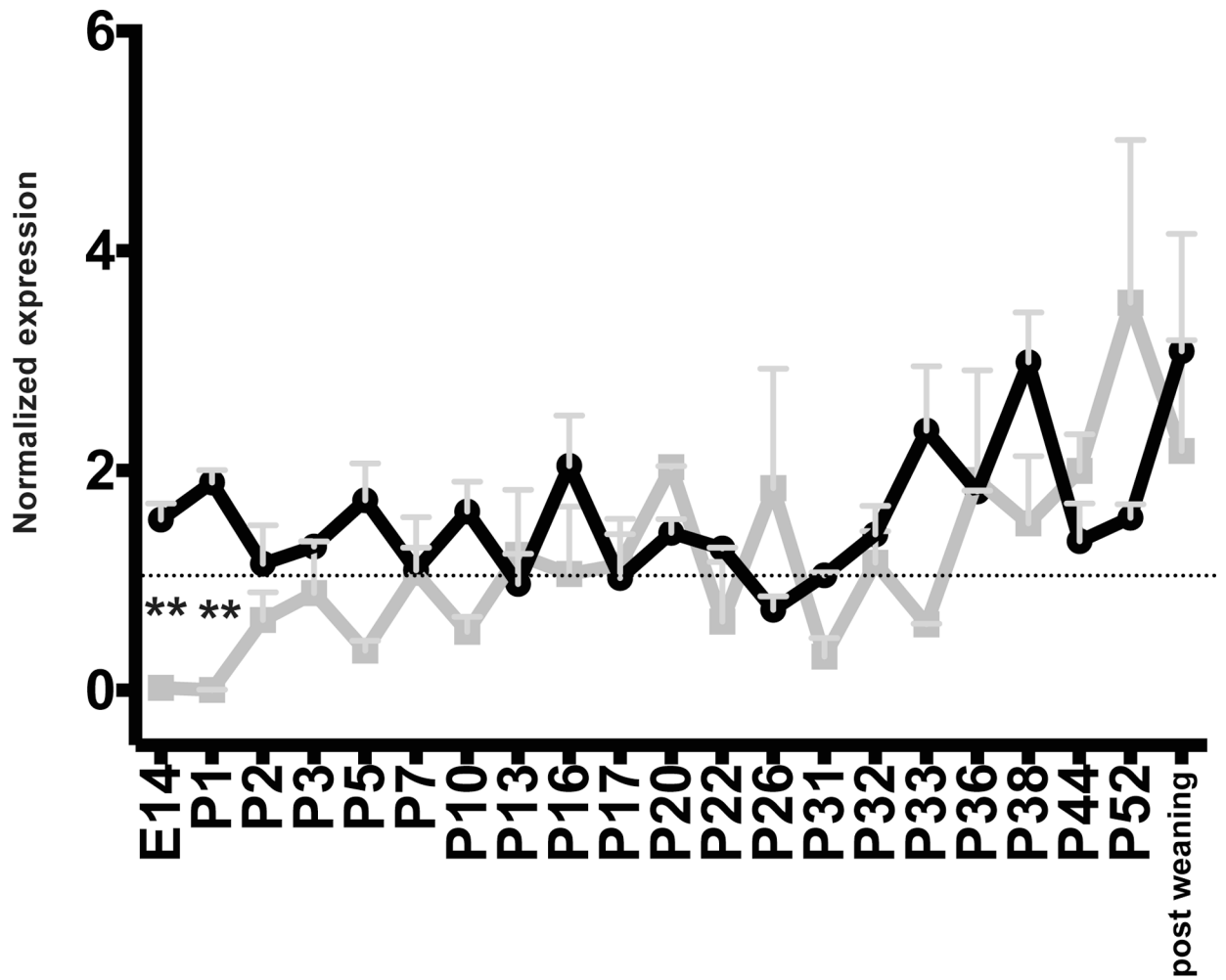
Figure 2: Abundance of IgA and pIgR transcripts appears to be highly coordinated throughout lactation.

IgA (gray line) and pIgR (black line) transcript abundance reported by day. Annotations as in Figure 1.
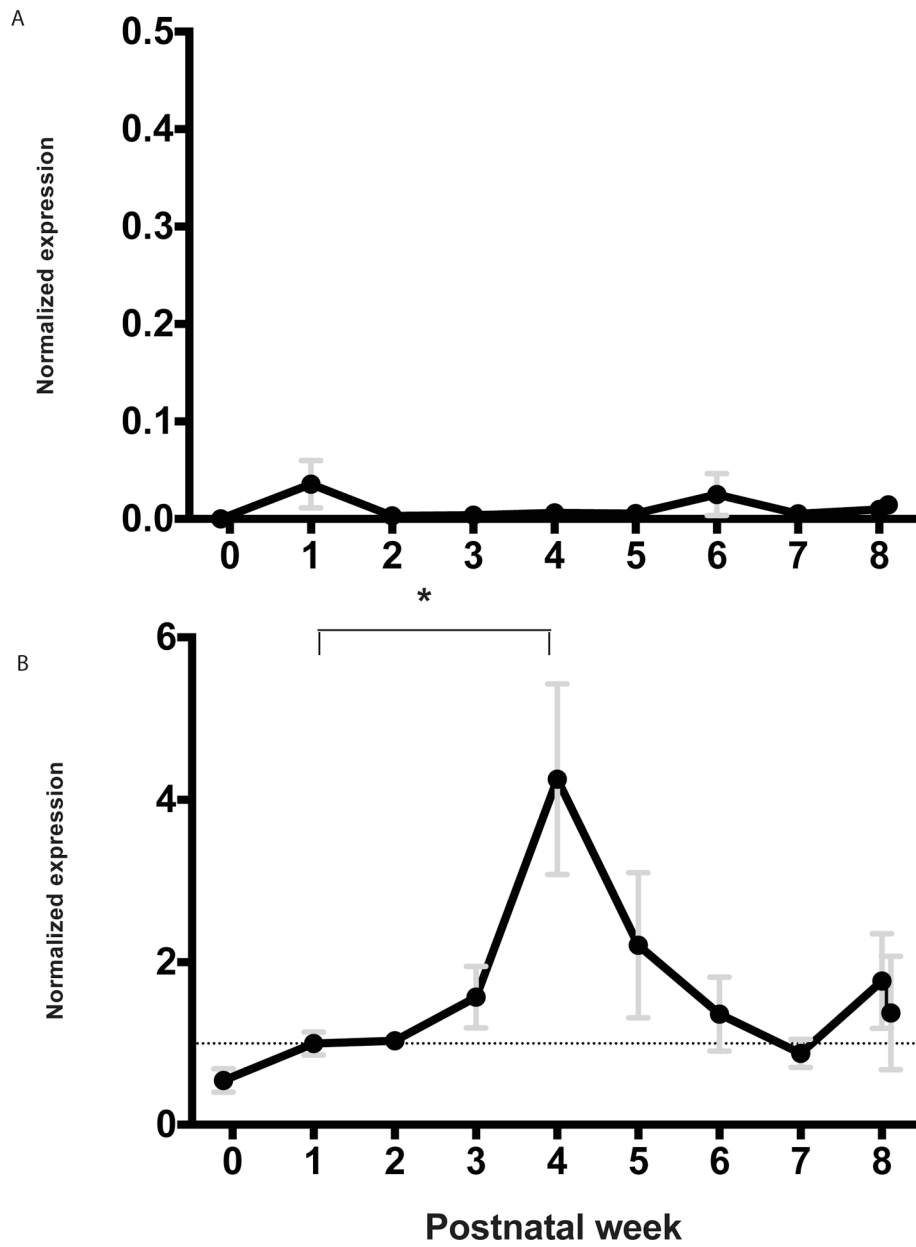
Figure 3: Normalized mammary transcript abundance of IgG (A) and FcRN (B).
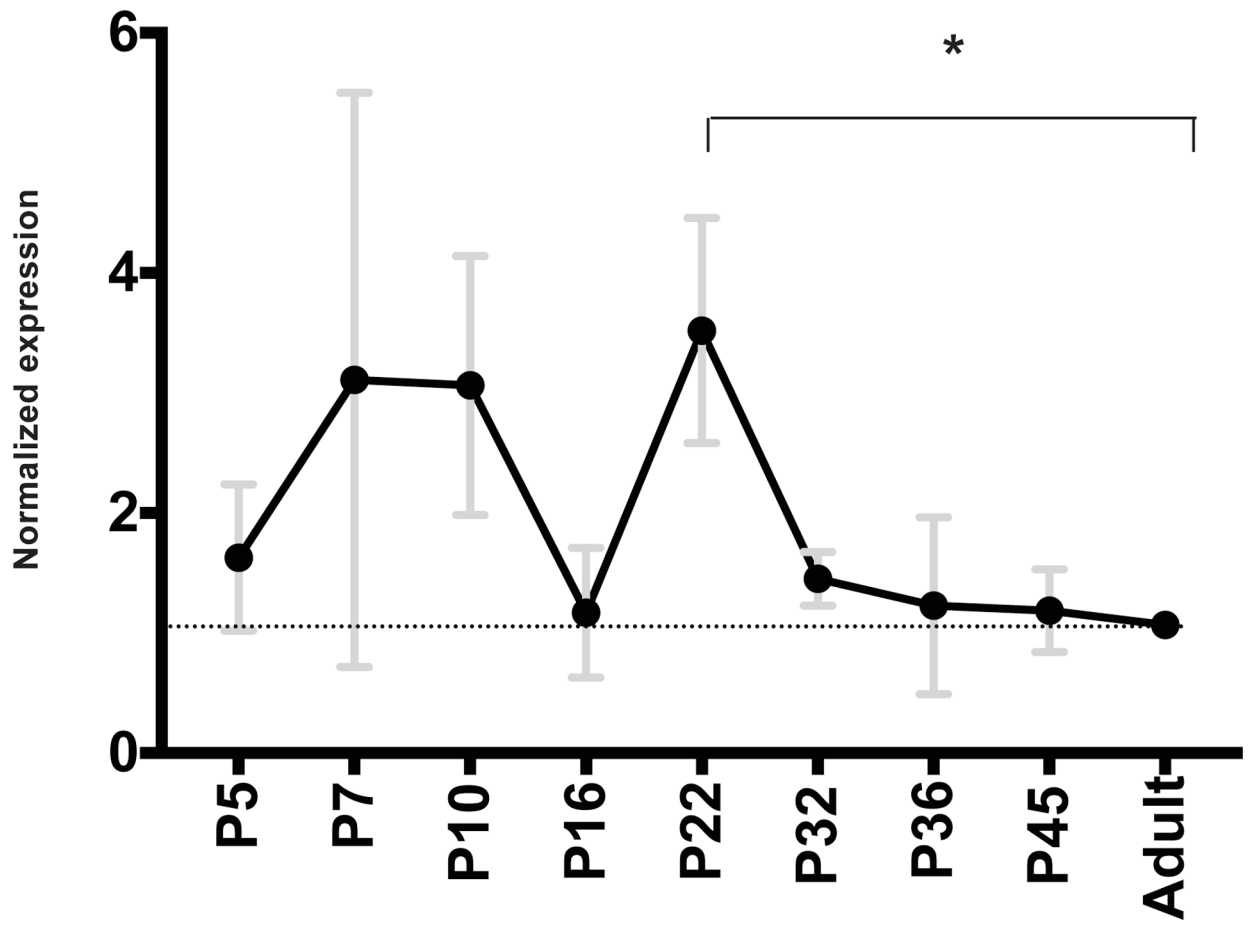Annotations and data pooling for weeks are as in Figure 1.

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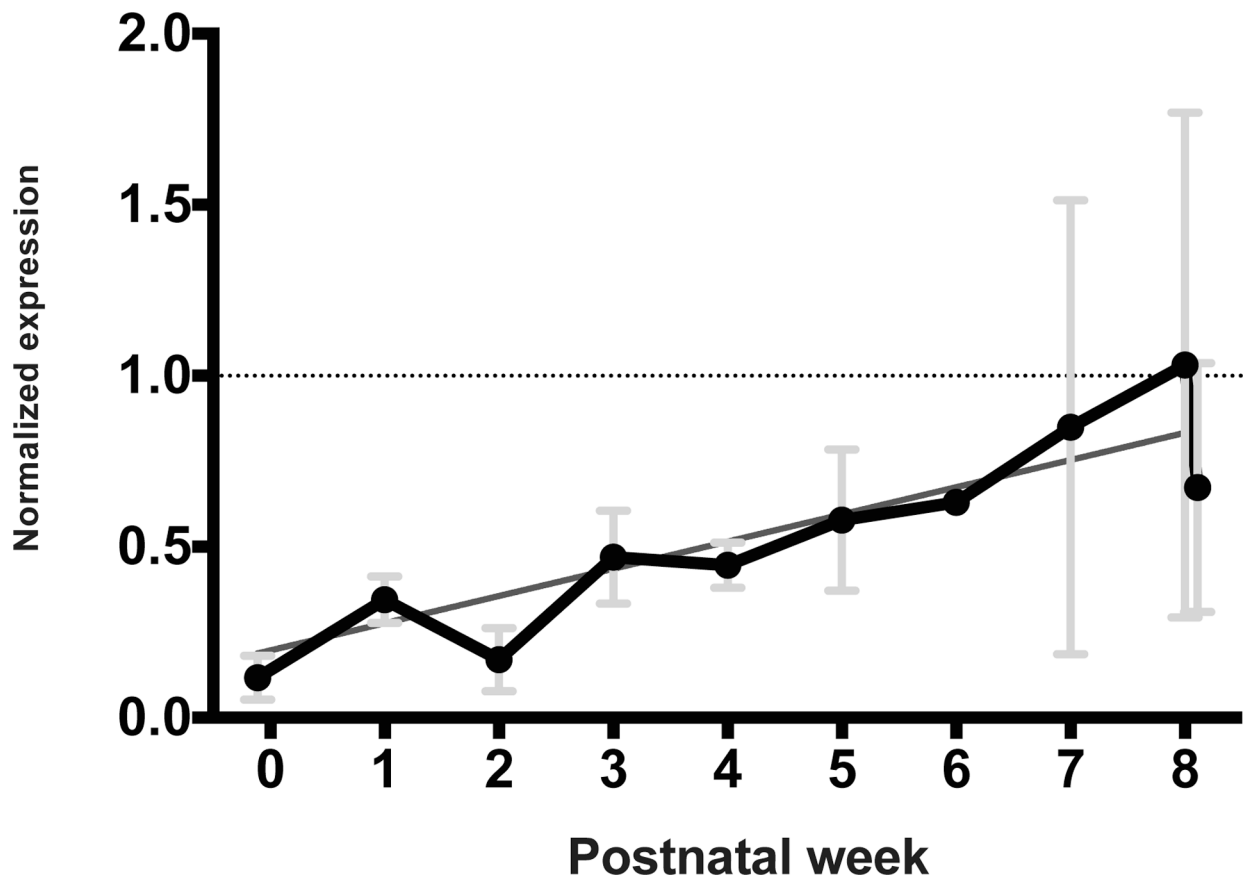Individual expression was normalized by reference gene ACTR2. Annotations as in Figure 1.

Figure 5: Normalized mammary transcript abundance of IgM.

Linear regression values for IgM were p = 0.0002, r^2 = 0.8373, slope = 0.08963.

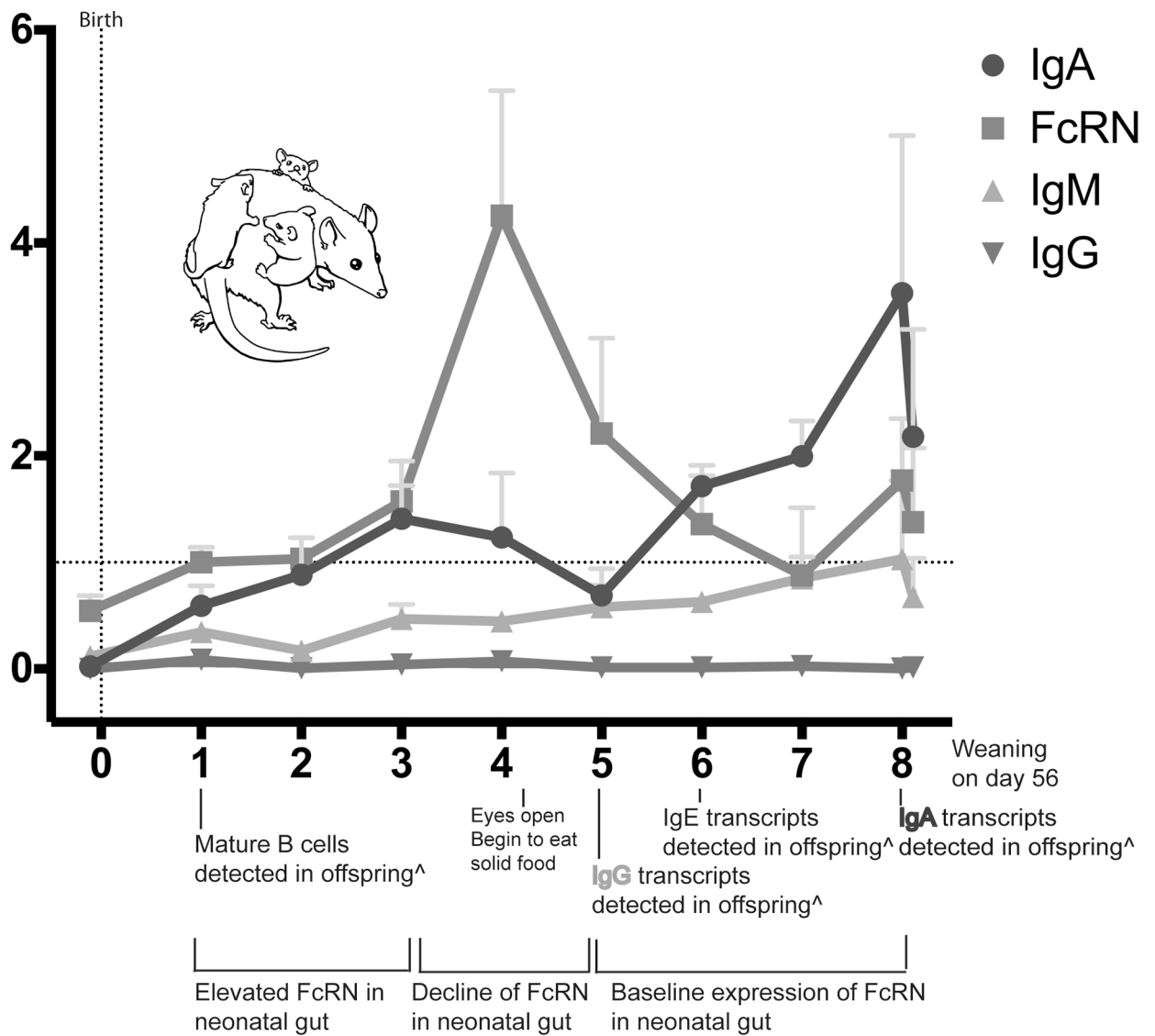Annotations and data pooling for weeks are as in Figure 1.

Figure 6: Immune related transcripts within the mammaries 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. âdapted from Wang et al 2012.