



Article

Early Deficient Lactation Differentially Affects Neonatal Thymic Cortical Development and Humoral Immune Responses in Rats

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Abstract

Hypoprolactinemia (hypoPRL) disrupts lactation and compromises milk production. Although maternal milk is a critical source of nutrients and bioactive compounds for newborns, the consequences of deficient lactation based on reduced milk quantity on the offspring's immune development remain incompletely understood. Therefore, this study aimed to elucidate how deficient lactation due to hypoPRL interferes with offspring immunity and development. Female Sprague Dawley (SD) and spontaneous hypoPRL Oncins France Colony A (OFA) rats were euthanized on day 2 of lactation to assess the impact of hypoPRL on serum, milk, and tissue samples. We demonstrated that early deficient lactation in the OFA model impaired maternal performance, leading to increased pup mortality during early lactation. OFA pups exhibited reduced body weight and weight gain, decreased cerebral weight and index, and an increased cephalization index. Thymic development was markedly altered, as evidenced by reduced thymus weight, area, and cortical extension. These structural changes were accompanied by increased thymic Rag 1 expression, suggesting altered lymphocyte development. In parallel, passive immune transfer was compromised, with reduced levels of OVA-specific immunoglobulin isotypes detected in pup serum, reflecting changes in milk-derived immune support. In summary, maternal hypoPRL during early lactation adversely affects offspring growth and thymic maturation with lasting consequences for neonatal immune maturation. This study provides a novel experimental framework to investigate the consequences of lactation



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deficiency with potential implications for understanding lactation insufficiency and its impact on neonatal immune exposure.

Keywords: prolactin; milk; thymus; antibodies; lactation; offspring

1. Introduction

Breastfeeding (BF) provides maternal milk from birth. The WHO recommends exclusive BF until 6 months of age, continuing with adequate complementary feeding up to 2 years of age [1]. However, despite recommendations, BF often ends earlier [2]. Maternal milk supplies nutritional components and a wide range of bioactive factors, including cells, hormones, immunoglobulins, enzymes, and growth factors [3], which enhance immunity, reducing infection risk and supporting intestinal homeostasis in newborns [3,4]. Colostrum is particularly important for early immune development, especially in T-cell ontogeny, due to its high concentration of immunologically active components with immediate protective effects [5,6].

Inadequate nutrition in infants due to a lack of exclusive BF during the first 6 months is associated with increased infant morbidity and mortality, mainly caused by diarrhea and acute respiratory infections [7]. It has been documented that newborns fed improperly have a smaller thymus, a finding that correlates with alterations in T-cell subpopulations [8], highlighting the relevance of adequate BF for optimal immune development.

The endocrine system plays a crucial role in the BF process [9,10]. Specifically, several hormones including prolactin (PRL), progesterone (P), estrogens (E), and thyroid hormones (THs) contribute to mammary development during pregnancy [11]. In addition, PRL and oxytocin are essential for milk synthesis and release during lactation [11]. While the endocrine regulation of lactation is well established [12], the specific effects of these hormones modulating immune responses within the mammary gland are still not fully understood.

PRL is a lactogenic peptide hormone that plays a key role in mammary epithelial development and milk synthesis [13]. During lactation, physiological hyperprolactinemia is required to maintain lactogenesis [11]. Conversely, hypoprolactinemia (hypoPRL) can compromise female reproductive health and BF success by reducing milk production (hypogalactia) and, consequently, impairing offspring nutrition [14]. This alteration may result from transient factors (medication, nicotine, obesity, and psychological factors) or from permanent conditions, such as pituitary hypofunction or genetic mutations [15].

To investigate the biological consequences of PRL deficiency under controlled conditions, we employ the Oncins France Colony A (OFA) rat strain, a model of spontaneous lactation deficiency linked to reduced circulating PRL levels after delivery and during lactation [16,17]. Previous studies have shown that OFA rats display elevated hypothalamic dopaminergic tone, which suppresses PRL release and consequently impairs lactation [17].

The deficiency of PRL could also impact the immune system [18], given its pleiotropic actions [19]. PRL directly exerts an immunomodulatory role by influencing T and B lymphocyte function, promoting proinflammatory responses, and regulating macrophage phagocytic processes [10]. During lactation, PRL contributes to the recruitment of immune cells to the mammary gland, enhancing the migration of CD4⁺ T lymphocytes, macrophages, eosinophils, and neutrophils in a murine model [13]. Consistently, PRL increases CCL2 and CXCL1 mRNA expression—chemokines involved in macrophages and neutrophils homing [13]. A previous laboratory study showed that PRL deficiency alters circulating lymphocytes and reduces T-cell recruitment to the mammary gland [18]. However, whether

PRL shapes maternal and neonatal humoral immune responses during lactation and whether it affects the development of the neonatal immune system remains unclear.

The focus of this work is aimed at elucidating how deficient lactation due to hypoPRL interferes with immunity and offspring development. Using the OFA rat model, which displays a partial blockade of the suckling stimulus impairing PRL secretion [18], we observed that hypoPRL adversely impacts maternal performance and neonatal survival as well as neonatal immune development.

2. Results

2.1. Hypoprolactinemia Impacts on Maternal Performance and Neonatal Survival

Since we have previously reported deficient lactation in hypoPRL rats [17], we evaluated maternal performance parameters in SD and OFA rats (Figure 1). Maternal PRL levels in serum at L2 were decreased in OFA rats with respect to SD (Figure 1A). Additionally, no changes in implantation sites (Figure 1B) or the number of offspring per litter (Figure 1C) were observed; however, OFA rats had a reduced ratio of offspring/implantation sites (Figure 1D). Pup mortality was higher than for SD animals on L1 (Figure 1E) and L2 (Figure 1F), but no changes in dam weight (Figure 1G) were observed between OFA and SD rats. The splenic weight (Figure 1H) and index (Figure 1I) were reduced in OFA rats, whereas the ovaric weight (Figure 1J) and index (Figure 1K) were increased. No changes in mesenteric lymph node weight (Figure 1L) or mesenteric lymph node index were reported (Figure 1M).

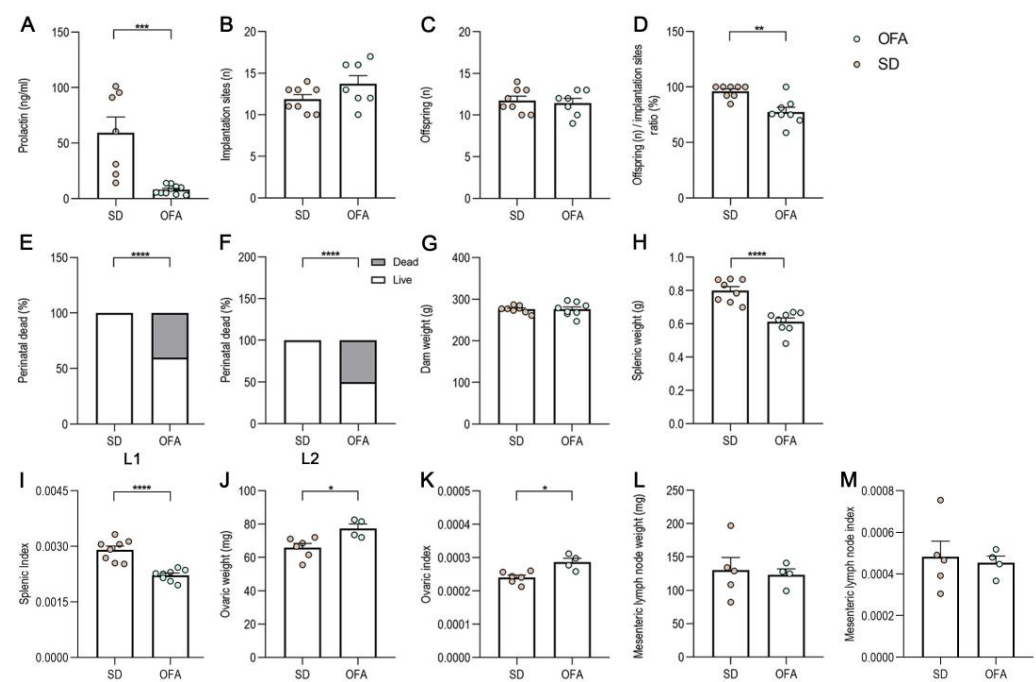


Figure 1. Maternal parameters in SD and OFA rats. Serum prolactin levels (A), number of implantation sites (B), number of pups (C), offspring/implantation site ratio (D), perinatal mortality at L1 and L2 (E,F), maternal body weight (G), spleen weight (H) and index (I), ovarian weight (J) and index (K), and mesenteric lymph node weight (L) and index (M). Each column represents the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.2. Early Deficient Lactation Impairs Offspring Development

The female and male pups of OFA dams weighed less on days 1 and 2, without growth between L1 and L2 (Figure 2A). Moreover, less weight gain (Figure 2B) and lower BMI (Figure 2C) were observed in female and male pups. No changes in length (Figure 2D), head

circumference (Figure 2E), or cerebral weight (Figure 2F) were observed. The cephalization index, a neurodevelopmental parameter, was increased in the OFA group (Figure 1G) in female and male pups. The cerebral index was lower in female and male OFA pups (Figure 1H).

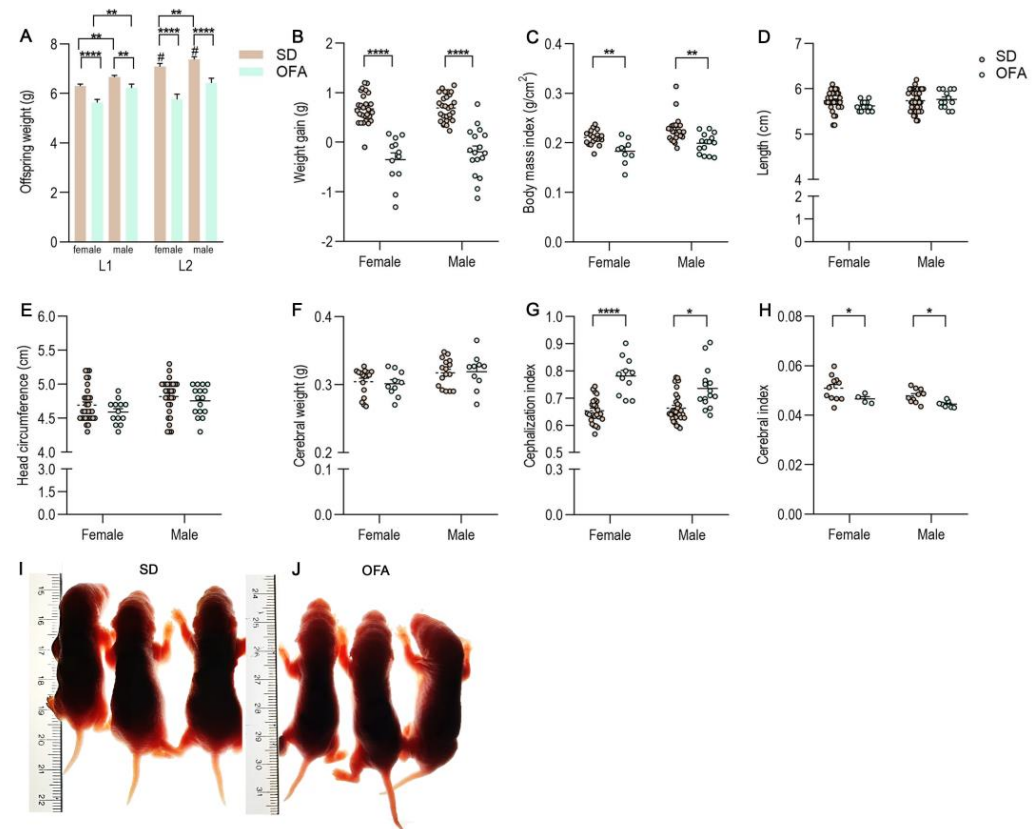


Figure 2. Postnatal growth and biometric parameters in SD and OFA pups. Weight at L1 and L2 (A), weight gain (B), body mass index (C), and body length (D) in the pups at L2. Head circumference (E), cerebral weight (F), cephalization index (G), and cerebral index (H). Representative photographs of offspring at postnatal day 2 from SD and OFA groups (I,J). Each orange/green circle represents individual values. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, # data differ between L1 and L2 within the same strain and sex.

2.3. Thymic Morphometry, Histology, and Rag1 Expression Are Altered in OFA Pups

In this context, thymic morphometric parameters were reduced in OFA compared with SD pups. Both females and males from the OFA group exhibited a significantly lower thymic weight (Figure 3A), male thymic index (Figure 3B), and thymic area (Figure 3C,D). Concerning histological analysis, a photo merge at 4X revealed no significant differences in medullary area between groups (Figure 3E). In contrast, the cortical area was markedly reduced in OFA offspring, both female and male, compared to their SD controls (Figure 3F). Consequently, the cortex-to-medulla ratio was significantly altered in OFA pups, indicating a relative reduction in the cortical section (Figure 3G).

Representative hematoxylin-and-eosin-stained photo-merged sections at 4X (Figure 3H) show preserved corticomedullary organization in all groups; however, OFA thymuses exhibited a thinner cortex and reduced cortical extension compared to those of SD pups.

At the molecular level, *Foxn1* mRNA expression showed no significant differences between groups (Figure 3I). In contrast, *Rag1* gene expression was markedly decreased in OFA females and males relative to SD rats (Figure 3J).

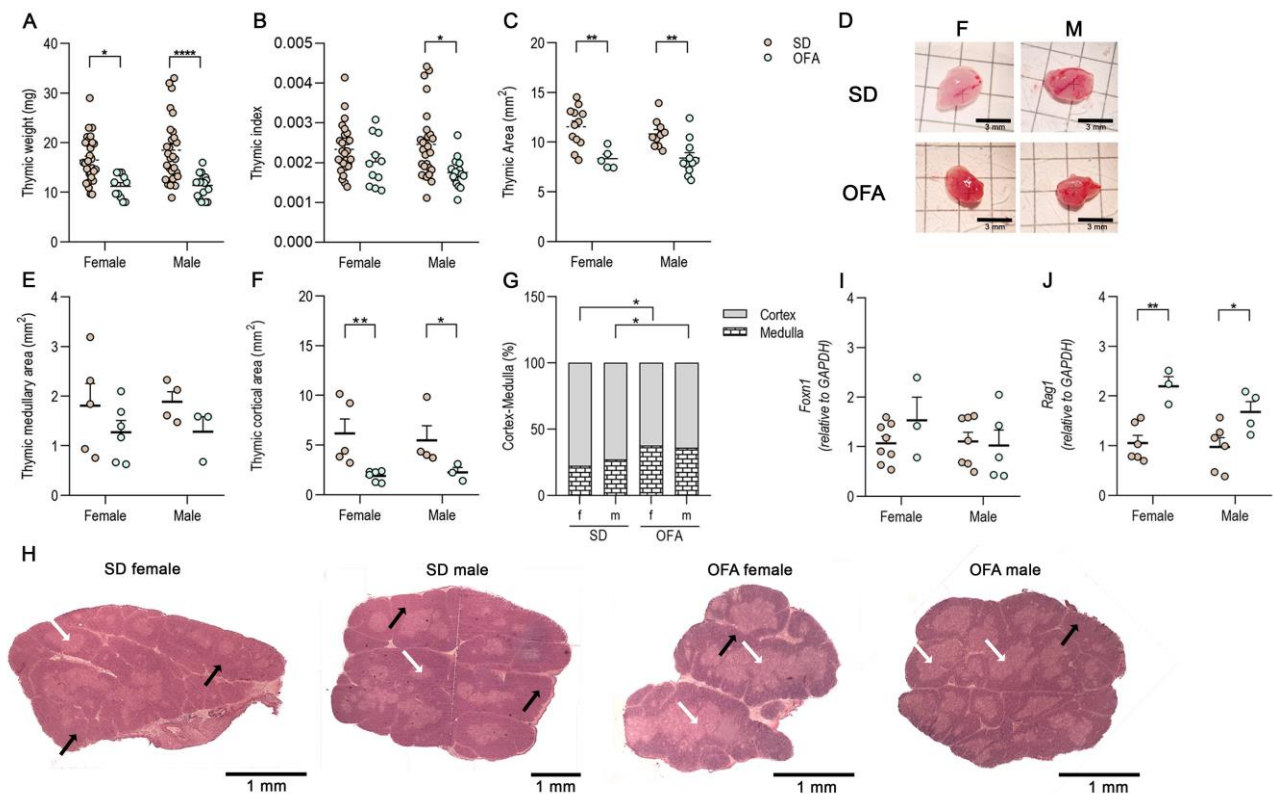


Figure 3. Thymic morphology, histology, and gene expression in SD and OFA offspring. Thymic weight (A), thymic index (B), and thymic area (C,D). Histological analysis of medullary (E) and cortical regions (F) and cortex-to-medulla ratio (G). Representative merge of thymic images at 4× showing cortical (black arrows) and medullary (white arrows) regions (H). Expression levels of Foxp1 (I) and Rag1 (J) (normalized to GAPDH). Each point represents individual values with the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

2.4. OFA Rats Present Altered IgG and IgG2a Levels in Serum

Immunohistochemical analysis from the mammary gland (Figure 4A) revealed no differences in average DAB intensity (Figure 4B) and number of IgA-positive cells per field (Figure 4C) between groups. OFA dams showed higher total serum IgG concentrations compared with SD controls (Figure 4D). However, no differences in milk or pup IgG levels (Figure 4E,F) were observed. Additionally, OFA dams exhibited higher serum IgG2a levels (Figure 4G), whereas milk concentrations were similar between groups (Figure 4H). Interestingly, OFA pups showed reduced IgG2a levels compared with SD pups (Figure 4I). Natural antibodies' anti-DNP-specific IgG responses were significantly increased in OFA dams (Figure 4J) and in their offspring (Figure 4K).

Together, these results suggest that the lactation-deficient OFA phenotype is associated with selectively altered maternal antibody profiles.

2.5. OFA Rats Present Early Defective Passive Immunity

OFA dams showed significantly lower OVA-specific IgG2a (Figure 5A) and IgG2c levels (Figure 5C) without differences in IgG2b (Figure 5B), IgM (Figure 5D), or IgA (Figure 5E) compared with SD dams. With respect to milk, OFA rats showed lower levels of OVA-specific IgG2a (Figure 5F), IgG2c (Figure 5H), and IgM (Figure 5I) without differences in IgG2b (Figure 5G) or IgA (Figure 5J). In offspring, OVA-specific IgG2a (Figure 5K), IgG2b (Figure 5L), IgG2c (Figure 5M), and IgM (Figure 5N) were markedly decreased in pups from OFA dams, demonstrating reduced passive acquisition of antigen-specific immunity.

OVA-specific IgA in pups was not detectable in either group, with all samples falling below the assay detection limit (Figure 5O).

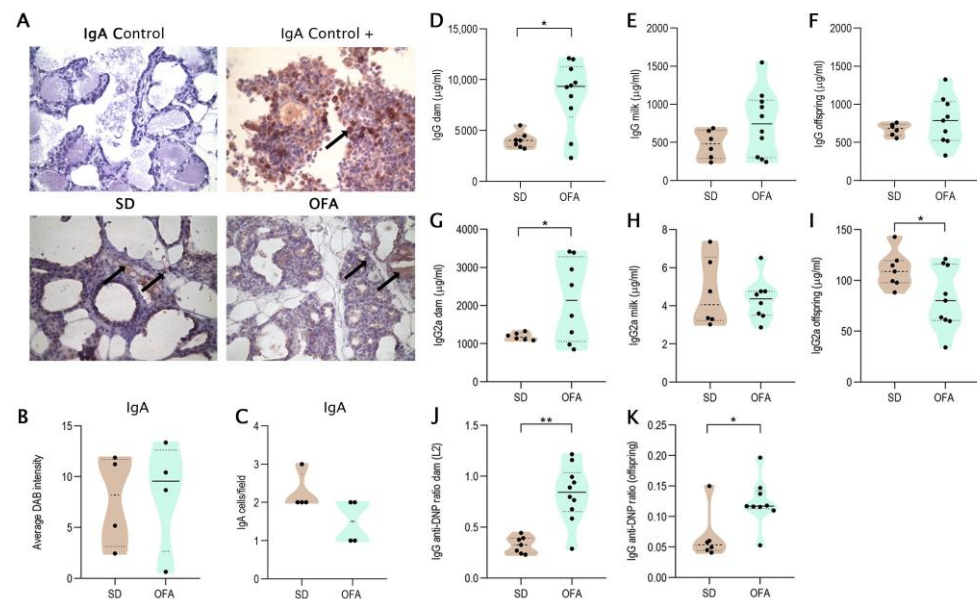


Figure 4. Maternal and pup antibody levels on L2. Micrographs of mammary gland IgA and positive control mesenteric lymph node (A). Mean IgA signal intensity (B) and IgA cells per field (C). IgG in maternal serum (D), milk (E), and pups (F). IgG2a in maternal serum (G), milk (H), and pups (I). IgG anti-DNP ratio in dam serum (J) and offspring serum (K). The results are presented as individual values with violin plots showing the distribution. The mean $\pm$ SEM is expressed. * $p < 0.05$, ** $p < 0.01$.

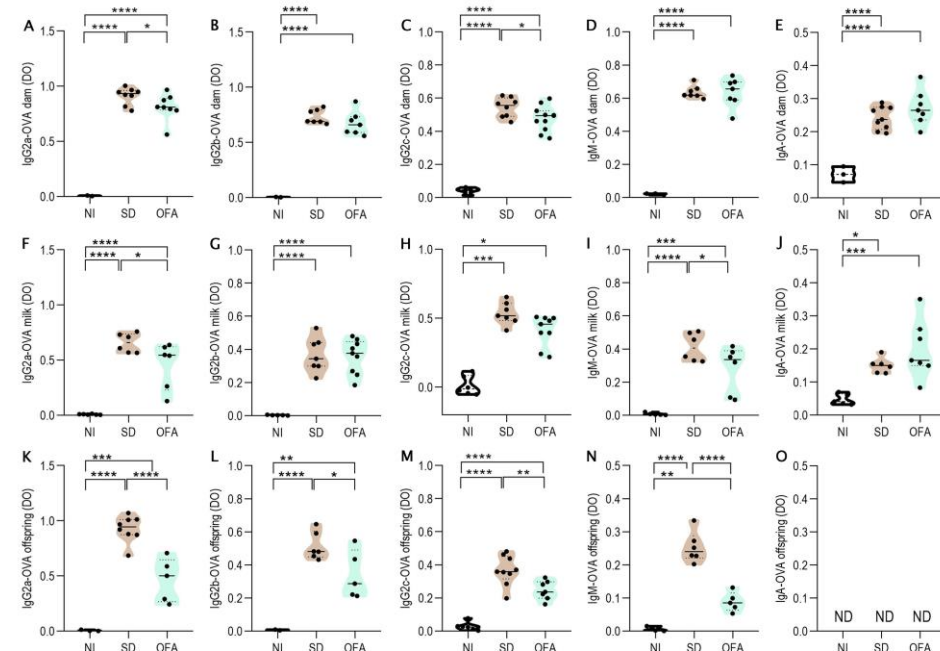


Figure 5. OVA-specific antibody responses were measured in serum and milk samples from SD and OFA dams and in the offspring's serum. Antibody isotypes were assessed as follows: in dam serum and dam milk—IgG2a (A,F), IgG2b (B,G), IgG2c (C,H), IgM (D,I), and IgA (E,J); in offspring serum—IgG2a (K), IgG2b (L), IgG2c (M), IgM (N), and IgA (O). Data for non-immunized controls (NI) are shown for both SD and OFA. The results are presented as individual values with violin plots showing the distribution. The mean $\pm$ SEM is given. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.6. Disrupted Coordination of Immunoglobulin Isotypes Across Maternal Serum, Milk, and Offspring OFA Conditions

Correlations corresponding to the same isotype measured across maternal serum, milk, and pup serum are highlighted by black boxes in the correlation matrices.

In the SD group, several significant correlations were observed across compartments (Figure 6A). In particular, correlations within the same isotype across maternal serum, milk, and pup serum were significant for IgM (positive correlation) and IgG2a (negative correlation), reflecting coordinated antibody profiles between mothers and offspring.

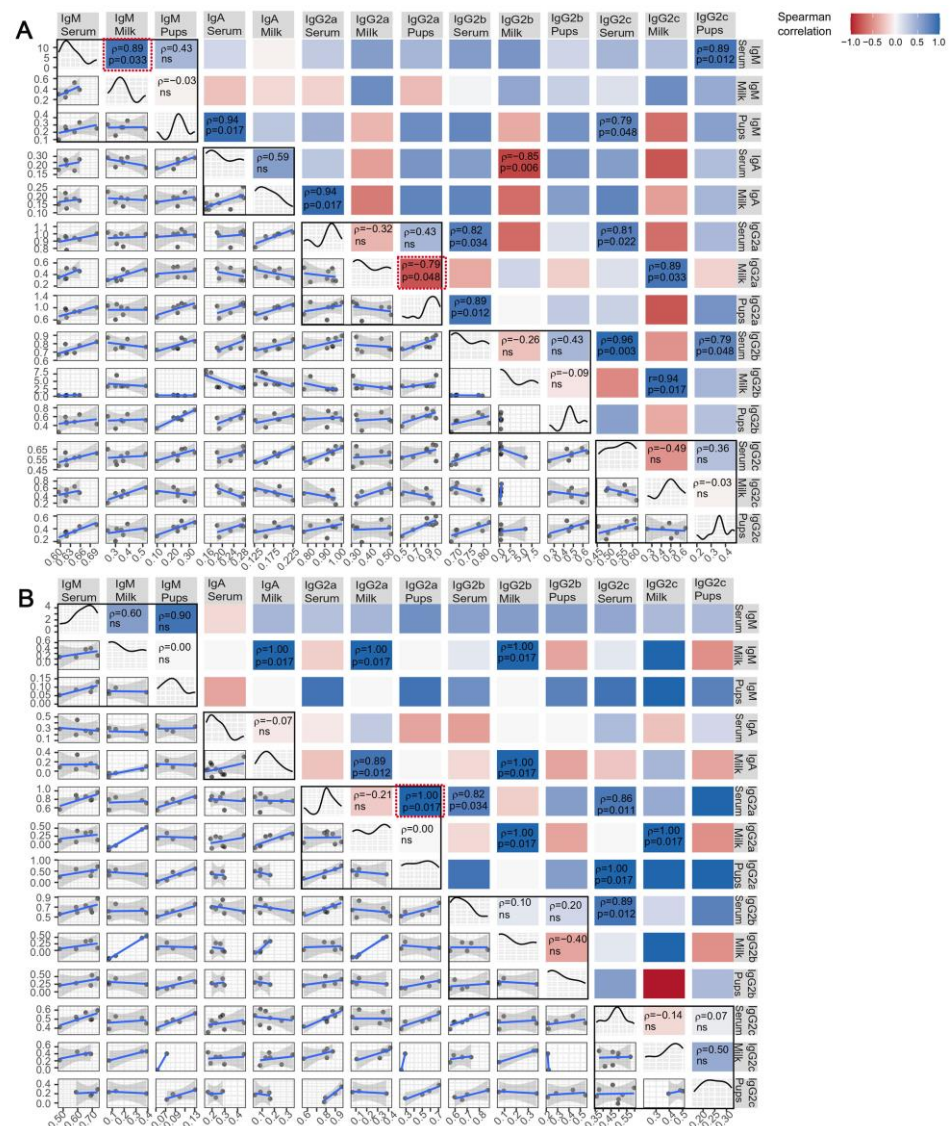


Figure 6. Correlation of immunoglobulin isotypes across maternal serum, milk, and offspring serum. Correlation matrices of immunoglobulin levels measured in maternal serum, milk, and pup serum in the SD (A) and OFA groups (B). Colored squares represent the direction and strength of associations. Correlations of the same immunoglobulin isotype across maternal serum, milk, and pup serum are highlighted by black boxes. Scatter plots illustrate individual correlations, and density plots are shown along the diagonal. Significant Spearman's correlations are indicated by the corresponding p values within each panel. Red frames indicate significant correlation between the same antibody isotype measured in different sample types.

In contrast, the OFA group exhibited a different pattern of correlation compared with SD controls (Figure 6B). Notably, correlations involving IgM showed a different profile

in OFA rats, with fewer significant associations between maternal serum, milk, and pup serum levels. Only IgG2a showed a positive correlation between pup and dam serum.

3. Discussion

In the present study, we describe the impact of hypoPRL-induced deficient lactation on maternal performance, offspring survival, early offspring development, and passive immune transfer. Using the OFA model, we broaden our knowledge about how early deficient lactation negatively affects development and immunological outcomes in the offspring. Our data further highlight the relevance of lactation as a critical window for nutritional and immune programming.

OFA dams exhibited impaired maternal performance accompanied by increased perinatal mortality, suggesting an inability to sustain neonatal demands during early lactation. Notably, previous studies have reported that OFA rats exhibit exacerbated stress responsiveness, which may contribute to their lactational deficit, as indicated by elevated corticosterone levels [16]. Consistent evidence from different experimental models indicates that increased corticosterone levels are associated with maternal stress and linked to reduced offspring viability, as shown in mice [20]. Consistent with these observations, our group has reported that pharmacological attenuation of stress responses with diazepam improves pup survival and restores the PRL response to suckling in the hypoPRL lactation-deficient OFA rats [16]. These alterations in the stress responses could represent an unfavorable physiological environment supplied to the offspring.

Rather than detailing individual biometric parameters, our results collectively indicate that offspring nursed by OFA dams exhibit early postnatal growth impairment consistent with malnutrition [21]. Alterations in morphometric indices associated with asymmetric growth further suggest that early PRL deficiency may affect developmental trajectories [22] during a highly vulnerable period. Previous studies have reported that hormonal imbalances during the intrauterine and early postnatal periods can adversely affect brain development [23], reducing cerebral weight and increasing the cephalization index [24], which has been associated with altered body symmetry, psychomotor retardation, and neurobehavioral disorders [25]. In line with these findings, PRL could play a critical role during early postnatal development by regulating milk composition. Supporting this notion, bromocriptine-treated dams, in which PRL secretion is pharmacologically suppressed, showed impaired offspring weight during early lactation [14]. Moreover, similar growth and morphometric alterations have been described in experimental models of hyperthyroidism [24], a condition that induces a hypoprolactinemic state, reinforcing the role of PRL deficiency in impaired early growth.

Beyond somatic growth, early-life PRL deficiency generated marked thymic alterations in OFA pups. Given the well-established sensitivity of the thymus to malnutrition [26,27], a condition known to induce thymic atrophy with preferential cortical depletion [28], the observed reduction in thymic mass together with the altered expression of genes involved in T-cell maturation suggests a disruption of normal thymic development. In this context, the observed upregulation of *Rag1* gene expression, a marker of immature thymocytes that is downregulated upon maturation [29], may reflect a compensatory response aimed at sustaining early differentiation under conditions of reduced thymic mass. The combination of increased *Rag1* expression and cortical reduction suggests a disruption of normal thymic maturation, driven by direct or indirect effects of the hormonal and nutritional alterations during early life. These findings support the idea that early nutritional and hormonal deficiencies may compromise the establishment of adaptive immune competence.

Given the immaturity of the neonatal immune system at birth, passive immune transfer through the placenta or milk is essential for early protection. Despite elevated total IgG

levels in OFA dams, offspring exhibited reduced levels of antigen-specific immunoglobulins, indicating an impairment in the quality rather than the quantity of humoral immune factors transferred through milk. Correlation analyses suggested coordinated maternal–offspring antibody transfer in control animals, an association that was disrupted in some factors in OFA litters. However, these correlations do not imply causality and should be interpreted cautiously, as additional maternal, endocrine, or developmental factors may contribute to the observed immune alterations. These results agree with previous studies showing that pups nursed by dams with elevated corticosterone levels exhibit reduced transfer of virus-specific IgG antibodies [20]. Furthermore, these correlations result in increased susceptibility and mortality following herpes virus infection, highlighting the impact of maternal stress hormones on the quality of passive immunity [20]. The selective reduction in specific antibody isotypes suggests that deficient lactation alters the quality of immune factors delivered through milk rather than reflecting a global failure of FcRn- or pIgR-mediated transport [30].

Although our milk obtention protocol is based on several reports using oxytocin and anesthetics [31,32], the potential influence of these treatments used during sample collection cannot be fully excluded and may have contributed to variability in the measured outcomes. While the OFA model provides valuable insight into the consequences of PRL-deficient lactation, its translational relevance should be considered with caution. Species-specific differences in lactation biology and immune development may limit direct extrapolation to humans. Nevertheless, the present findings highlight that endocrine alterations during lactation can influence early growth and immune programming, with potential implications for understanding how disrupted lactational environments may affect offspring health across species.

In conclusion, maternal hypoPRL during early lactation disrupts milk-mediated immune support and thymic development, leading to impaired growth and compromised neonatal immune maturation. These findings emphasize the critical role of optimal early lactation in neonatal immune development and passive immune protection. Future studies should explore the long-term immunological consequences of early lactation impairment and evaluate potential interventions for lactation disorders with translational relevance.

4. Materials and Methods

4.1. Animals and Experimental Protocol

The animals were treated according to the Guiding Principles in the Care and Use of Animals of the U.S. National Institutes of Health. All procedures were approved by the Institutional Animal Care and Use Committee of the Facultad de Ciencias Médicas, Universidad Nacional de Cuyo (Protocol 226/2022). We used twelve-week-old Sprague Dawley (SD) and Oncins France colony A (OFA hr/hr) female rats bred in our laboratory. The animals were housed under standard conditions, with a controlled temperature and light cycle (lights on from 6:00 a.m. to 8:00 p.m.). Water and food were available *ad libitum*. Estrous cycles were monitored daily using vaginal cytology, and only females exhibiting regular 4- or 5-day cycling were used.

Nineteen females were included and randomly distributed to the control group (SD rats, $n = 9$) and hypoPRL group (OFA rats $n = 10$). Females were mated nine days after starting cycling. The presence of spermatozoa in vaginal smears the morning after mating was considered day 0 of pregnancy. On lactation day 1 (L1), litter size was adjusted to 8 pups per dam, 4 male and 4 female, whenever possible.

On lactation day 2 (L2), the dams were separated from their pups for 3 h to allow for milk collection. The dams were anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg) [33] and subsequently injected intraperitoneally with 2 IU of oxytocin (10 IU/mL

Synkro). Twenty minutes later, milk samples were collected using gentle manual pressing of selected mammary nipples. Approximately 0.5–1 mL of milk was obtained per dam and stored at -80°C until analysis.

Following milk collection, the dams were euthanized via decapitation. Trunk blood was collected, and serum was obtained using centrifugation at $960\times g$ for 15 min and stored at -20°C . The spleen, ovaries, and mesenteric lymph nodes were dissected and weighed using an analytical balance (Sartorius, Göttingen, Germany). The mammary glands were fixed in 4% formaldehyde (Biopack, Buenos Aires, Argentina) for immunohistochemical analysis. All procedures were performed during the light phase between 10:00 and 11:00 a.m. Pups were sexed on L1 by measuring the anogenital distance and weighed on L1 and L2 using an analytical balance. Weight gain (g) was calculated as the difference between body weight on L2 and L1 and was analyzed in SD pups (29 females, 32 males) and OFA pups (13 females, 17 males). On L2, body length and head circumference were measured with a caliper before euthanasia via decapitation. Subsequently, brain weight was recorded in SD (17 females, 16 males) and OFA (11 females, 11 males) and thymic weights and thymic area were recorded using an analytical balance and magnifying glass (Zeiss, Oberkochen, Germany), respectively (SD, 12 females and 10 males; OFA, 6 females and 11 males). For histological analysis, thymuses were fixed in 4% formaldehyde (Biopack, Buenos Aires, Argentina). Pup serum was obtained using centrifugation of trunk blood at $960\times g$ for 15 min and stored at -20°C .

The cephalization index was calculated as the ratio of head circumference (cm) to body weight (g) [34] in SD (11 females, 10 males) and OFA (4 females, 8 males). Organ indices were calculated as organ weight relative to body weight, and body mass index (BMI) was calculated as body weight (g) divided by body length squared (cm^2). Additionally, the offspring-to-implantation site ratio was determined in SD (29 females, 32 males) and OFA (13 females, 17 males).

4.2. OVA Sensitization Protocol

To induce a specific humoral immune response, SD and OFA dams were randomly selected and immunized with ovalbumin (OVA; $30\text{ }\mu\text{g}$ per $100\text{ }\mu\text{L}$ /animal) (Sigma Lot 89C-0146 N $^{\circ}$ A-5253, St. Louis, MO, USA). Subcutaneous injections were administered in the dorso-lumbar region. The animals received OVA emulsified in incomplete Freund's adjuvant (Sigma, N $^{\circ}$ F-5506 Lot 117F-8985, St. Louis, MO, USA) seven days before mating, followed by a booster injection on gestational day 1.

4.3. Hormonal Determination

Serum PRL levels were determined in the morning on lactation day 2 between 10:00 am and 11:00 a.m., using a double-antibody radioimmunoassay (RIA) following extraction with diethyl ether as previously described [16,35]. All samples were run in duplicate. The intra-assay coefficient of variation was $<5\%$, and the inter-assay coefficient of variation was $<10\%$. The lower limit of detection was 0.5 ng/mL .

4.4. Histological and Immunohistochemical Analysis

Thymuses were fixed in 4% formaldehyde and included in paraffin blocks. Serial sections ($3\text{--}5\text{ }\mu\text{m}$) were obtained using a microtome Leica SM2000R (Leica Microsystems, Wetzlar, Germany) and stained with hematoxylin and eosin to evaluate thymic histoarchitecture. Thymus samples from 18 offspring were analyzed ($n = 5$ SD females, $n = 6$ OFA females, $n = 4$ SD males, and $n = 3$ OFA males). Slides were visualized using light microscopy Nikon E200 (Nikon Instruments Inc., Tokyo, Japan) and digital images were acquired at $4\times$ magnification. The total cortical and medullary areas (mm^2) were quantified using Image J software (version 1.43, National Institutes of Health, Bethesda, MD, USA).

Five mammary glands per experimental group were fixed with formaldehyde at 4% and included in paraffin blocks. For IgA immunohistochemistry detection, antigen unmasking was carried out in 0.01 M citrate buffer (pH 6.0) at 100 °C for 15 min. Sections were incubated overnight at 4 °C in a humidity chamber with a goat anti-rat IgA primary antibody (R-9630 Lot 083K4826, Sigma, St. Louis, MO, USA) at a dilution of 1:1500. After, sections were incubated with biotinylated rabbit anti-goat IgG secondary antibody at a dilution of 1:100 (E0466, Dako, Glostrup, Denmark) followed by the avidin–biotin ABC complex (VECTASTAIN Elite ABC System, Vector, Burlingame, CA, USA). Immunoreactivity was visualized using diaminobenzidine (DAB; Vector, USA) as a chromogen. Sections were lightly counterstained with hematoxylin and observed using an Eclipse E400 light microscope (Nikon Instruments Inc., Tokyo, Japan). Rat mesenteric lymph nodes were used as positive controls, whereas mesenteric lymph nodes and mammary gland sections processed in parallel without the primary antibody were included as negative controls.

Digital images were analyzed using Image J software (version 1.43, National Institutes of Health, Bethesda, MD, USA). The number of cells per field and median and mean pixel intensities were quantified at 40×. For each analyzed animal, measurements were performed in 10 randomly selected fields, and the final value corresponds to the mean of the measurements obtained from these fields. For background correction, an unstained area of each section was selected and analyzed; values were expected to be close to 255. When necessary, background subtraction was applied. Color deconvolution was performed using the H-DAB vector, and the DAB channel was selected for thresholding. The average pixel intensity of the threshold image was used as a measure of the IgA-positive staining area.

4.5. RNA Purification and Real-Time PCR Analysis

To evaluate the expression of genes essential for thymic development and function, thymic epithelial cell activity, and lymphocyte maturation (*Foxn1* and *Rag1*), thymuses were collected from four groups: 8 SD females, 7 SD males, 3 OFA females, and 6 OFA males. Samples were stored at −80 °C and subsequently homogenized in 500 µL of TRI Reagent (Genbiotech Store, Buenos Aires, Argentina). Total RNA was extracted following the manufacturer's instructions. The RNA concentration was determined spectrophotometrically using a Nano-500 Micro Spectrophotometer (Hangzhou All sheng Instruments Co., Ltd., Hangzhou, China).

Two micrograms of total RNA were reverse-transcribed with MMLV Reverse Transcriptase (Promega, Madison, WI, USA) and random hexamer primers (Promega, Madison, WI, USA) in a 20 µL reaction. mRNA abundance was quantified via real-time PCR using Agilent Technologies Stratagene Mx3005P and HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis Biodyne, Tartu, Estonia). The following rat-specific primers were used (Genbiotech Store, Buenos Aires, Argentina): GAPDH (forward: AGACAGCCGCATCTTCTTGT; reverse: CTTGCCGTGGGTAGAGTCAT) [36]; *Foxn1* (forward: GGGCTTCAAAATTCCCTCG; reverse: CAAGACCAAGAGCCTACCCC); and *Rag1* (forward ACATTGTTGTGTGGGTGGGT and reverse: CTGTTGCTCTTCTGGCCTCA) [37].

Real-time quantification involved measuring the fluorescence increase from EvaGreen (Biotium, Fremont, CA, USA) binding to double-stranded DNA at each cycle's end. Gene expression was analyzed with the software MxPro version Mx3005P using the $2^{-\Delta\Delta C_t}$ method [38], with GAPDH as the reference gene. All samples were run in duplicate.

The PCR cycling conditions were as follows: For *Foxn1* and *Rag1*, initial activation at 95 °C for 12 min; 40 cycles of denaturation at 95 °C for 15 s; annealing at 60–65 °C for 20 s; and extension at 72 °C for 20 s. For GAPDH, initial activation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min; and final extension at 72 °C for 5 min.

4.6. Immunoglobulin Analysis

Because impaired lactation may compromise the passive transfer of maternal antibodies, total IgA, IgG, and IgG2a levels, as well as natural anti-DNP-specific IgG levels, were evaluated in dams, milk, and offspring serum. In addition, OVA-specific immunoglobulins were assessed in dams, milk, and offspring following maternal immunization to further evaluate antibody transfer during early lactation. Immunoglobulin levels in milk and maternal and pup serum were quantified using an enzyme-linked immunosorbent assay (ELISA). High-binding 96-well plates (Greiner bio-one, Frickenhausen, Germany) were coated overnight at 4 °C with capture antibody diluted in PBS, followed by blocking with PBS–1% gelatin solution (for 1 h at 37 °C in a humidity chamber).

Samples and IgG and IgG2a standards (BD Pharmingen, San Jose, CA, USA) were incubated for 2 h at room temperature in a humidity chamber. IgG was detected using an HRP-conjugated secondary antibody, whereas IgG2a was detected using a biotinylated secondary antibody followed by the appropriate conjugate. The conjugates were incubated for 1 h at 37 °C. After washing, the enzymatic reaction was revealed by the addition of the substrate solution (12.5 mL of 0.1 M Sodium Acetate pH 5.5, 200 µL of 6 mg/mL 3,3',5,5'-tetramethylbenzidine in dimethylsulfoxide, and 50 µL of 1% H₂O₂). Color was allowed to develop in the dark at room temperature and stopped with H₂SO₄ 1N at the times indicated by the manufacturer. Optical density (OD) was measured at 450 nm using a microplate spectrophotometer LabSystems, Multiskan MS (Thermo LabSystems, Helsinki, Finland).

Total immunoglobulin levels were determined in dam serum (SD $n = 8$ –9; OFA $n = 7$ –10), dam milk (SD $n = 6$ –8; OFA $n = 7$ –10), and offspring serum (SD $n = 8$ –9; OFA $n = 5$ –7), depending on the immunoglobulin isotype analyzed. Total IgG and natural anti-DNP IgG levels were evaluated in dams (SD $n = 7$; OFA $n = 10$), whereas IgG2a anti-DNP antibodies were assessed in SD ($n = 7$) and OFA ($n = 9$) dams.

OVA-specific antibodies were determined by coating ELISA plates overnight at 4 °C with OVA (Sigma Lot 89C-0146 N° A-5253) at a concentration of 10 µg/mL in PBS. After blocking, serum or milk samples were added in serial dilutions and incubated for 2 h at room temperature. Serum samples from SD and OFA non-immunized dams were included as negative controls ($n = 3$ –6 per group). Bound OVA-specific immunoglobulin isotypes and subclasses were detected using mouse monoclonal anti-rat antibodies: IgG2a (clone R2A-2, Sigma R-0761 Lot 103H4850, St. Louis, MO, USA), IgM (clone RTM-32 Sigma R-0886 Lot 81H4840, St. Louis, MO, USA), IgG2c (clone MARG2c-3 Sigma R-6888 Lot 126H4806, St. Louis, MO, USA), and IgG2b (clone R2B-8 Sigma R-1011 Lot 81H4816, St. Louis, MO, USA). Detection was performed using an HRP-conjugated goat anti-mouse IgG secondary antibody. Plates were developed with TMB substrate as described above, and OD was read at 450 nm.

Natural IgG anti-DNP antibodies were also assessed using the same ELISA protocol described for OVA-specific antibodies. The only modification was the coating of plates with DNP-BSA (2 µg/mL) in PBS.

The capture antibodies used for total immunoglobulin quantification included mouse anti-rat IgG2a (BD Pharmingen), IgM, IgG2b, and IgG2c (Sigma), as well as goat anti-rat IgG (Sigma-Aldrich, St. Louis, MO, USA). For detection, biotinylated mouse anti-rat IgG2a (BD Pharmingen) and HRP-conjugated rabbit anti-rat IgG (Sigma-Aldrich) were used as appropriate.

4.7. Statistical Analysis

The data distribution was assessed using the Shapiro–Wilk test. Comparisons between two groups were performed using Student's *t*-test when the data followed a normal

distribution. For comparisons involving more than two groups, a two-way analysis of variance (ANOVA) was applied when assumptions of normality were met, considering sex and strain as fixed factors, followed by Fisher's LSD post hoc test. The results of the two-way ANOVA, including the main effects and interaction terms (sex $\times$ strain), are presented in Supplementary Table S1. When the sample size was smaller than five ($n < 5$) or when the data did not meet the assumption of normality, the non-parametric Mann–Whitney U test was used for pairwise comparisons. Comparisons involving more than two groups were analyzed using the Kruskal–Wallis test followed by Dunn's post hoc test. For the OVA-specific antibody ELISA assays, Spearman's correlation analyses were conducted to evaluate relationships among different antibody isotypes and subclasses in pup serum, maternal serum, and milk. Data are presented as the mean $\pm$ SEM. A p value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software (version 8.0.1 for Windows; GraphPad Software, San Diego, CA, USA).

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27041708/s1>.

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Abbreviations

The following abbreviations are used in this manuscript:

BF	Breastfeeding
PRL	Prolactin
P	Progesterone
E	Estrogens
THs	Thyroid hormones

HypoPRL	Hypoprolactinemia
L1	Lactation day 1
L2	Lactation day 2
BMI	Body mass index
OVA	Ovalbumin

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