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Glucocorticoid regulation of milk production, lactose synthesis, and alpha-lactalbumin gene expression

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Glucocorticoid regulation of milk production, lactose synthesis, and α-lactalbumin gene expression

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DISSERTATION

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I joined the Hovey lab for my MD/PhD training because I wanted to become a comparative mammary gland biologist so that, ultimately, I could use translational research methods to develop the evidence-base for lactation medicine. Dr. Russ Hovey and Dr. Josie Trott spent innumerable hours, through blood, sweat, tears, and probably even some milk, teaching me the fundamentals of science: from metaphorically "putting my head inside of a tube to think about the chemistry of the reaction" to building up basic molecular biology skills required for experimentation. I am leaving this well-rounded PhD training experience in the Hovey lab with an unparalleled exposure to a range of *in vivo* and *ex vivo* experimental models in comparative mammary gland biology. As a testament to Dr. Hovey's dedication to my professional growth as an independent scientist, I must acknowledge the grants that I have received thanks to his mentorship: National Institutes of Health NICHD F30HD101295, UC Davis Daniel T. O'Connor MD Memorial Research Grant, UC Davis Clinical and Translational Sciences Center Pre-Doctoral TL1 Fellowship UL1 TR001860 and linked award TL1 TR001861, and the UC Davis College of Agricultural and Environmental Studies Henry A. Jastro Research Award. I would be remiss not to acknowledge the friendship and encouragement provided by fellow PhD and MD/PhD students Carmen Banks, Alice Mathews, Shasta McMillen, Brendan Cohn-Sheehy, Catherine Lee, Annica Stull-Lane, and Monica Keiko Lieng. I am also grateful to the members of my thesis committee—Dr. Danielle Lemay and Dr. J. David Furlow—for their insights and to my MD/PhD mentors—Dr. Saul Schaefer, Dr. Caroline Chantry, Dr. Lars Berglund, and Dr. Nicholas Kenyon—for their advice. I want to thank my parents for coming up to Sacramento from San Jose nearly every weekend since the birth of my son in 2018 to support me while I complete the PhD requirements. Most importantly, I need to thank my husband for being my cheerleader every step of the way. I dedicate this dissertation to him.

ABSTRACT

The most common reason for premature breastfeeding cessation is real, or perceived, low milk supply. Maternal stress suppresses milk production, but the mechanism by which stress inhibits lactation has not been defined. The mammalian stress response is mediated in large part by glucocorticoids (GC). It is well-established that excessive endogenous and exogenous GCs transiently suppress milk volume and milk lactose content, without affecting milk fat or protein levels. *In vitro* studies from the 1980s suggested that the biphasic regulation of α-lactalbumin (LALBA) synthesis by GCs is central to the pathophysiology of stress-induced suppression of milk lactose content and milk yield. The LALBA protein is unique to the mammary gland and is required for lactose synthesis and milk production, yet its transcriptional regulation has yet to be defined.

We first sought to define the effect of a synthetic GC (dexamethasone, DEX) on milk yield and composition alongside changes in mammary gene expression in dairy cows. We demonstrated that a single, high dose of DEX administered to lactating dairy cows transiently suppressed milk volume, milk lactose content, milk LALBA content, and *LALBA* gene expression. Then, we wanted to determine the reliability and replicability of the murine mammary explant system for the study of GC-mediated regulation of *Lalba* expression. We defined doses of corticosterone (CORT) and DEX and time intervals at which *Lalba* expression could be maximally upregulated, inhibited, or suppressed following initial stimulation. We demonstrated for the first time that *Lalba* expression in mammary explants from midpregnant mice can be suppressed by over 50% in response to high dose of CORT after initial 48 h of stimulation by a low dose of CORT.

The extent to which a decrease in LALBA gene and protein expression contributes to the decline in milk lactose content and milk yield remains to be determined. Future research endeavors should focus on defining the GC-regulated transcriptional landscape at the *Lalba* promoter using the murine mammary explant system. Determining the mechanism by which GCs

modulate LALBA transcription will inform preventative and therapeutic strategies for low milk supply and improve maternal-child health outcomes.

Chapter 1. A comparative review of the cell biology, biochemistry, and genetics of lactose synthesis

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ABSTRACT

Lactose is the primary carbohydrate in the milk of most mammals and is unique in that it is only synthesized by epithelial cells in the mammary glands. Lactose is also essential for the development and nutrition of infants. Across species, the concentration of lactose in milk holds a strong positive correlation with overall milk volume. Additionally, there is a range of examples where the onset of lactose synthesis as well as the content of lactose in milk varies between species and throughout a lactation. Despite this diversity, the precursors, genes, proteins and ions that regulate lactose synthesis have not received the depth of study they likely deserve relative to the significance of this simple and abundant molecule. Through this review, our objective is to highlight the requirements for lactose synthesis at the biochemical, cellular and temporal levels through a comparative approach. This overview also serves as the prelude to a companion review describing the dietary, hormonal, molecular, and genetic factors that regulate lactose synthesis.

INTRODUCTION

Milk is essential for mammalian survival, where lactose is one of its major components that is synthesized and secreted by the mammary epithelium, either in its free form or as an oligosaccharide. The concentration of lactose in milk is strongly correlated with the overall volume of milk output. As such, defining the mechanisms that underlie lactose synthesis represents a first step in developing strategies to manipulate and improve the production and composition of milk.

Herein we review the various precursors, genes, proteins, and ions required for optimal lactose synthesis. In doing so, one of our primary objectives is to highlight ways in which various mammals, from marsupials to marine placental therians, ruminants and non-ruminant livestock, rodents, and primates have retained or adapted mechanisms for lactose synthesis to meet the demands of their environment and the needs of their offspring. By reviewing the physiology, biochemistry, and genetics of lactose synthesis through a comparative lens, we also aim to provide a background for the second part of this review where we further explore the extrinsic and intrinsic factors that regulate lactose synthesis.

The importance and variability of lactose in milk

One of the most fascinating aspects of milk produced by mammals is the range of its lactose concentration [1]. Free lactose, a disaccharide comprised of glucose and galactose, is the primary carbohydrate in the milk of most placental mammals and comprises more than 80% of all its carbohydrate [2,3]. Consistently, across nearly all mammalian species, there is a negative relationship between the content of lactose and fat in milk (Fig 1), which ensures the offspring receives a steady source of calories [1]. For example, human infants receive 40% of their caloric requirements from the approximately 70 g/d of lactose they consume in the first six months of life [4]. In fact, the concentration of lactose in the milk of different primates is consistently high (61 to 89 mg/ml) and accounts for one- to two-thirds of total milk energy [5]. In a similar way, lactose in bovine milk (44 to 56 mg/ml) provides approximately 30% of the calories required by newborn

calves [1,6] . Milk produced by rodents has a lower content of lactose, ranging from 24 to 28 mg/ml in mice, and 11 to 41 mg/ml in rats [7]. Aquatic Pinnipeds, like the otariid species, produce milk devoid of lactose that enables them to avoid involution induced by milk stasis [8], while others like the phocids, vary their production of lactose [9–12]. This broad diversity in the lactose content of milk across species remains an underexplored information treasure trove, not only for better understanding the physiological regulation of lactose, but also for refining an understanding of how nutrients are partitioned across a range of demands and tissues in support of lactation.

Lactose also acts as the primer for oligosaccharide synthesis [13], where it serves as the reducing end of the oligosaccharide core [14]. The >130 different forms of oligosaccharides in human milk serve as prebiotics for the growth of beneficial bacteria in the infant gut [3,15]. The abundance of these oligosaccharides and their abundance in milk relative to lactose varies widely across species. For example, the ratio of oligosaccharides to lactose in human milk is between 0.21 and 0.38, where the oligosaccharide content decreases from 23 mg/ml in colostrum to between 5 and 12 mg/ml in mature milk [16]. By contrast, bovine milk contains around 39 oligosaccharides at a concentration from 0.03 to 0.06 mg/ml, which is comparable to that in ovine milk (0.02 to 0.04 mg/ml), albeit lower than that in caprine milk (0.25 to 0.3 mg/ml) [3]. The milk of mice and rats contains a variety of oligosaccharides, where milk from rats, for example, contains more sulfated oligosaccharides [13,17]. Even though the oligosaccharide content of milk has been characterized for dozens of species [13], the role of these oligosaccharides during neonatal growth and development in non-human mammals remains poorly understood [3,18,19].

Oligosaccharides are the primary carbohydrate in the milk of monotremes, marsupials, and in many carnivores, especially among the Arctoidea species (except the domesticated dog) where the ratio of oligosaccharides to free lactose is considerably higher than that found in human or bovine milk. For example, the ratio of oligosaccharides to free lactose ranges from 7:1 for the striped skunk and 5:1 for mink to 31:1 for polar bears and 52:1 for the Japanese bear [18,20]. When it comes to monotremes and marsupials, the lactose in their milk (~18 mg/ml) is

secreted in a form of tri- and tetra-saccharides or galactosyl oligosaccharides, respectively [13], with the majority of monotreme lactose bound to a fucosyl group [21]. Furthermore, marsupials alter the ratio of oligosaccharides to lactose in their milk across a lactation [22–24]. As an example, the milk of the Tammar wallaby contains approximately 25 mg/ml lactose, which rises to around 39 mg/ml after 13 weeks. After 28 weeks, the lactose in their milk is cleaved within the mammary epithelium, thereby eliminating any free lactose from the milk for the remaining lactation. As a result of these changes the content of oligosaccharides and free glucose and galactose in the milk of the Tammar wallaby increases as its free lactose content declines [25].

The synthesis of lactose by the mammary gland is also a major determinant of its milk volume output, where the concentration of lactose in milk is positively associated with its volume and negatively associated with the osmolarity of its salts [1]. A hypothetical model proposed in the 1970-80's described the swelling of Golgi vesicles with water in response to lactose synthesis and accumulation, thereby offsetting the high osmotic potential of lactose as the consequence of its multiple hydroxyl groups [26]. Specifically, hydrogen bonds form between the hydroxyl groups of lactose and a molecule of water, whereas the ring oxygens and the bridging oxygen in lactose do not bind water [27]. The length of this hydrogen bond (fructose < sucrose < glucose < lactose ≪ mannose) in mono- and disaccharides is also negatively correlated with the sweetness of the carbohydrate [27]. Lactose has more opportunities for hydrogen binding and hydration than the inorganic salts in milk (i.e., Cl, Na), which explains why in most species the lactose concentration of milk is inversely correlated with its osmolality and positively correlated with milk volume. The inverse correlation between the concentration of lactose and inorganic salts in milk also maintains milk as isosmotic to blood [1], which is essential for sustained and optimal milk synthesis.

These unique biochemical properties of lactose highlight the important contribution that this seemingly simple carbohydrate makes to the nutritive value of milk, alongside its crucial role in the sustained transfer of essential water and solutes to the offspring. As a changing climate

and reduced water availability threaten mammalian survival, there becomes a greater need to understand the critical role for lactose in milk across a range of species. However, given that laboratory mice and rats each produce different sets of oligosaccharides in their milk, as well as compared to human milk, the question must be raised as to which model organism is best-suited for studying lactose and oligosaccharide synthesis. In a related way, it is worth emphasizing that genetic selection for milk production in livestock has likely also shifted the relative abundance of lactose in milk, albeit the extent of such changes historically can be difficult to assess. The best evidence for such a shift comes from a long-range genetic selection study in Holstein dairy cows, where selection for production traits reflective of the modern industry saw cows have a higher lactose content in their milk, mostly during late lactation [28].

PRECURSORS, GENES, AND PROTEINS REQUIRED FOR LACTOSE SYNTHESIS

Lactose precursors and mammary hexoneogenesis

There is no doubt that lactose synthesis creates massive pressure on an animal's metabolic balance, where the partitioning of maternal nutrients in support of lactation, coined "homeorhesis," is critical to support ongoing milk synthesis [29]. To emphasize this point, the mammary glands of a dairy cow in peak lactation can use up to 85% of all circulating plasma glucose, where total glucose turnover can exceed 3 kg/d. Of the glucose that is taken up by the udder, between 65 and 70% is used to synthesize lactose [29]. Ultimately it is the mammary gland(s) that control glucose uptake and utilization from the circulation, although many questions remain as to precisely how they regulate the uptake of glucose in support of lactose synthesis [30,31]. The effect of plasma glucose levels on lactose synthesis is further explored in our companion review [32].

Precursors delivered to mammary epithelial cells (MEC) in support of lactose synthesis primarily originate from plasma glucose, where gluconeogenesis by the liver plays an important role in maintaining plasma glucose levels. However, we must emphasize that plasma glucose is

not the sole precursor for lactose synthesis, and that glycerol and galactose are alternative carbon sources for lactose synthesis. In fact, the contribution of plasma glucose to lactose synthesis during fed and fasting states is consistently in the order of 80 and 60%, respectively [33–41]. Dietary or infused glucose contributed to approximately 80% of the lactose synthesized by non-fasted lactating humans, but to only 62% of the lactose synthesized in the fasted state [36,40]. In the fed state, ≥ 98% of the glucose in lactose came from the plasma, whereas only 68% of uridine galactose (UDP)-galactose originated from plasma glucose. After a 24 hour (h) fast, 72% of the glucose and 51% of the UDP-galactose in human milk were derived from plasma glucose [36,42]. Similarly, approximately 70% of the lactose produced by both high and low producing goats was derived from plasma glucose [34], which was similar to the incorporation of plasma glucose into 59% of lactose carbon produced by sows [43].

One alternative source of carbon for lactose synthesis is glycerol that can be taken up directly by MEC and converted to glucose and UDP-galactose *de novo.* Glycerol accounted for approximately 14-70% of the UDP-galactose synthesized *de novo,* and approximately 10% of newly-synthesized glucose in fed and fasted lactating humans [36,40,42]. Likewise, in fed and starved goats, 27% and 21% of the UDP-galactose moiety of lactose, respectively, was created *de novo* from glucose 6-phosphate, which resulted in the asymmetric labeling of its carbon [40,44]. This process, coined mammary “hexoneogenesis,” generates hexose phosphates within MEC by integrating non-glucose precursors into the triose isomerase reaction or the pentose phosphate pathway [40,45]. In the case of the triose isomerase reaction in MEC, glycerokinase phosphorylates glycerol, which is then converted to dihydroxyacetone phosphate by glycerol-3-phosphate-dehydrogenase. Dihydroxyacetone phosphate then feeds directly into the triose isomerase reaction within the glycolytic pathway (Fig 2). A labeled precursor was more likely to have been recycled through the pentose phosphate pathway if the labeled glucose or UDP-galactose moieties within lactose had a higher C6-C4:C3-C1 ratio of enrichment [40,44]. By

contrast, when the glucose moiety in lactose is derived directly from plasma glucose, the distribution of its carbons is identical to that found in plasma glucose [37–41,46].

In a similar way, galactose can also be taken up by MEC for its direct incorporation into lactose. Infusion of ^{13}C-galactose into breastfeeding women yielded two ^{13}C peaks in milk lactose, one within hours and the second a day later due to the incorporation of ^{13}C-glucose derived from hepatic metabolism of ^{13}C-galactose. Only the C1-atom of galactose and the C1-atom of glucose in lactose were labeled, suggesting that infused, labeled galactose contributed to both the glucose and UDP-galactose moieties [36,42]. The direct incorporation of labeled plasma galactose into lactose indicates that MEC take up and route galactose to the Golgi, potentially through glucose transporter 1 (GLUT1) and UDP-galactose translocator (SLC35A2) [30,36,42].

These considerations regarding the supply and utilization of large quantities of substrate in support of lactose synthesis have broad implications for understanding the regulation and coordination of lactation physiology. Whereas the normal level of demand for glucose is already high, metabolic dysregulation during states ranging from obesity to undernutrition can quickly have a negative impact on lactation performance, while extreme states such as ketoacidosis can be fatal. Differential utilization of the various substrates during fed and fasted states highlights the need to further study how the endocrine environment regulates precursor mobilization as well as delivery to, and uptake by, the MEC. In essence, there is the need to refine our understanding of the factors controlling homeorhesis. As a step in this direction, we summarize some of the key hormones implicated in the regulation of lactose synthesis in our accompanying review [32].

THE CELL BIOLOGY OF LACTOSE SYNTHESIS

Lactose is produced exclusively in the Golgi apparatus of the MEC. Here we outline the pathway for lactose synthesis, assuming that all its carbon derives from circulating glucose. Extracellular glucose is taken up by MEC via GLUT1 and sodium-glucose transporter 1 (SGLT1),

then transported into the Golgi apparatus via GLUT1 (Fig 2) [30]. Glucose is then phosphorylated by hexokinase (HK) to yield glucose-6-phosphate, which is then used to create a pool of UDP-bound galactose in the cytoplasm. Several sequential steps then facilitate the *de novo* synthesis of UDP-galactose. First, phosphoglucomutase (PGM1-3) transfers a phosphate group from the C6 position of glucose-6-phosphate to the C1 position of glucose. Next, UDP-glucose-pyrophosphorylase (UGP2) exchanges the phosphate group for a UDP moiety. The resulting UDP-glucose is then converted to UDP-galactose by galactose epimerase (GALE). Alternatively, glucose-1-phosphate can first be converted to galactose via galactose epimerase (GALE), followed by the transfer of UDP to galactose-1-phosphate by galactose-1-uridyltransferase (GALT). UDP-galactose is then shuttled into the Golgi via SLC35A2 or SLC35B1 [47–49]. Within the Golgi apparatus, the final step of lactose synthesis occurs, where lactose synthase (LS) joins glucose and UDP-galactose by a β-1-4 glycosidic bond [50–52]. Importantly, LS is a unique enzyme complex comprised of β-1,4-galactosyltransferase-1 (B4GALT1) and the mammary-specific modifier protein α-lactalbumin (LALBA), and requires close association with the uridine nucleotide cycle on the trans-Golgi [53]. Once lactose is produced by LS, it is then packaged into vesicles from the *trans*-Golgi and transported to the apical membrane for exocytosis. With an eye to defining the control points for lactose synthesis, we characterize the individual proteins of the LS in the following sections, as well as the factors that regulate their interaction and activity.

The ubiquitous enzyme, B4GALT1

β-1,4-galactosyltransferases (B4GALT) belong to a family of seven transmembrane proteins that are present in most secretory cells in the body. In the absence of LALBA, these Mn-dependent enzymes transfer D-galactose from UDP-galactose to N-acetylglucosamine [54]. Central to LS activity is B4GALT1, whose structure, function, and orthology across species has been reviewed extensively [54–56]. The B4GALT1 protein (Fig 3) has two metal ion binding sites (sites 1 and 2), an N-terminal domain that recognizes the nucleotide donor (UDP-galactose), a C-terminal domain that recognizes the glucose acceptor, and an active site located between the

two domains. The amino-terminus of the mature B4GALT1 protein is embedded within the Golgi membrane and requires that Mn be bound to site 1 (Fig 3) for maximal activity [57]. This binding of Mn is an absolute requirement for the binding of UDP-galactose [58]. Site 2 is a low-affinity site that binds Mn or Ca, which serves the primary role of enhancing the efficiency of catalysis and the binding of glucose.

While the binding of Mn to site 1 of B4GALT1 is required for lactose synthesis to proceed (Fig 3), high concentrations of MnCl are inhibitory [57,59]. The activity of B4GALT1 was submaximal when ions such as Zn, Fe, Co were bound at site 1, while other ions including Na, K, Mg, and Ca did not affect B4GALT1 activity, as they could not bind site 1 [60]. It is the occupation of site 2 that determines the affinity of B4GALT1 for glucose [58]. The binding of Ca, Mg, ciopene, or spermidine to site 2 modified the ability of the enzyme system to synthesize lactose, emphasizing the complex role of B4GALT1 as a multi-ligand allosteric enzyme. Depending on which cation was bound to site 2, the K_m for Mn at site 1 could be lowered and the V_{max} raised, leading to the stabilization of glucose binding to LS [58].

The unique modifier protein, LALBA

Central to the activity and function of LS is LALBA, a protein that is expressed exclusively by MEC whose role has been reviewed extensively by others [61–63]. We should emphasize that beyond this role, LALBA secreted into milk is also important for infant nutrition due to its high tryptophan, lysine, and branched chain amino acid content [64]. In fact, human milk has one of the highest concentrations of LALBA (~2.4 mg/ml) which comprises more than a quarter of all its protein [64–66]. Perhaps not surprisingly, several lines of evidence also indicate that bioactive peptides derived from LALBA support maturation of the infant gut [64].

Variation in LALBA distribution

While we provide a more detailed overview of the transcriptional regulation of LALBA gene expression in the accompanying review [32], it is worth highlighting here that from a physiological context, the expression and distribution of LALBA within the mammary gland(s) is not as

homogenous as might be implied in the general literature. Indeed, others have highlighted that heterogeneity in the expression of LALBA mRNA and protein in the sheep and goat udder can misrepresent the true level of LALBA gene activation and protein synthesis [67,68]. *In situ* hybridization on biopsies from the udder of a 14 day prepartum ewe revealed that *LALBA* mRNA was expressed heterogeneously in isolated single MEC, in clusters, and in some lobules [69]. Likewise, epithelial cells within collapsed alveoli contained few to no milk fat globules but expressed high levels of *LALBA* mRNA, whereas MEC that contained abundant milk fat globules did not express *LALBA* mRNA. The *LALBA* mRNA transcript was also rarely detected in multilayered ducts such as in the gland cistern [69]. Similar transcriptional heterogeneity was also observed in the mammary glands of mice, where *LALBA* mRNA expression was uniform across the alveoli unless they were distended and the epithelium was flattened, or after the teats were sealed and milk stasis ensued. Notably, the expression of whey acidic protein (WAP) or β-casein mRNAs was not as heterogeneous [70], further highlighting that LALBA and other milk proteins are not expressed in perfect unison. As another demonstration of this heterogeneity, a comparison of total RNA from milk fat to that from biopsied mammary tissue or shed epithelial cells revealed that the abundance of LALBA transcripts was greater in the former [71]. In a similar way, Carli et al. recently identified that among MEC shed into human milk there were many functionally-distinct cells (>35%) that they proposed might sub-specialize in lactose production, while others (4%) were suggested as being primarily responsible for the synthesis of milk proteins and lipids [72]. We suggest it is more likely that this differential expression of lactose, protein and lipid synthesis across MEC reflects the heterogeneity and acute temporal regulation of milk synthesis in the gland at any given time. That said, a long list of knowledge gaps remains regarding factors that might regulate the transcription and translation of LALBA across individual MEC, whether that be cell stretch, local feedback, or local blood flow, to name but a few. Regardless, we should stress that an appreciation for the heterogeneity of LALBA expression

requires careful consideration when studying the physiological and local factors that regulate lactose synthesis.

The structure and function of the LALBA protein

Mature LALBA, which functions as an enzyme modifier, is a glycosylated 123 amino acid metalloprotein with a molecular size of ~14.5-18.5 kilodaltons. Its structure consists of a large α domain and a small β domain with a deep middle cleft that binds Ca [73,74]. In keeping with the essential role of LALBA across species, parts of its sequence are highly conserved; specifically, there are thirty invariant amino acid residues in LALBA across all mammals, most of which are involved with binding B4GALT1 and Ca [21,73]. A more detailed description of the role for various amino acids in the form and function of LALBA has been offered elsewhere [75,76].

The LALBA molecule has several critical regions. Saccharide binding is achieved through the cleft region of LALBA, which is homologous to that in C-type lysozymes. The aromatic cluster I (AC1) within LALBA, specifically Leu-110, facilitates its interaction with B4GALT1 and is critical for LS activity [73,74,77]. The crucial nature of this AC1 site is highlighted by the fact that mutation of the AC1/flexible loop region (as occurs in otariids like the fur seal) or deletion (as occurs in the walrus) suppresses LALBA synthesis and leads to a milk devoid of lactose [10,11]. The least flexible region of LALBA is the Ca-binding loop and a hydrophobic region, where residues Lys-79, Asp-82, Asp-84, Asp-87, and Asp-88, along with two molecules of water, support Ca binding [73,77]. This Ca-binding loop is formed by one disulfide bond between residues 73 and 91, with additional stability provided by residues 61 and 77 [73,77]. The number of Ca binding sites on LALBA varies by species as described elsewhere [74,78]. Likewise, a full review of metal ion binding to LALBA has been provided by others [76,78,79]

Post-translational modification of LALBA

The LALBA protein is often glycosylated, yet the biological significance of this post-translational modification is poorly understood (Fig 2). Importantly, both glycosylated and non-glycosylated forms of LALBA are active in LS and are secreted into the microsomal fraction of

milk (Fig 2) [80]. While there is abundant evidence in other protein and cell systems that N-glycosylation affects protein localization, stability, folding, and solubility, it remains unclear why LALBA is variably glycosylated [81]. Approximately 10% of bovine and murine LALBA is glycosylated, while only 1% of human LALBA is glycosylated [82–85]. In cats, the glycosylated and non-glycosylated forms of LALBA occur in equal ratios, whereas in rabbits LALBA is predominantly glycosylated [86,87]. Rat LALBA is unique in that it is present in milk as three charged forms, each of which is glycosylated [88]. Compared to bovine LALBA, rat LALBA has four amino acid substitutions (Lys/Glu at 43, Asp/Asn at 44, Gly/Gln at 46, and Glu/Asp at 49), which likely facilitates its increased glycosylation. Moreover, the secondary structure of the peptide sequence required for N-glycosylation in rat LALBA was predicted to have a ß-bent conformation, which would further enable B4GALT1 to access the glycosylation site [88]. The site of glycosylation in human LALBA is disputed, as glycosylation has been variably detected at the Asn45 site and at amino acid 71 (Asn-71-Ile-Cys), which is an amino acid triplet that is conserved in all LALBA except that of the red-necked wallaby [83,89,90]. Interestingly, when human LALBA was overexpressed in the udder of dairy cows, the transgene product was not glycosylated, whereas the endogenous bovine LALBA became unusually glycosylated at Asn-71 [91]. How these forms of LALBA differentially modulate lactose synthesis, or how the extent of glycosylation is regulated, still remains unclear.

Factors regulating the interaction between LALBA and B4GALT1

The interaction between LALBA and B4GALT1, which occurs in the Golgi apparatus in a specific order, is critical for LS activity and involves specific metal ions (Fig 3). Importantly, we should point out that *in vitro* studies of metal ion binding to LALBA were performed in the absence of B4GALT1, leaving it unclear as to whether the multiple conformations of LALBA described *in vitro* also occur *in vivo*, along with questions regarding the relevance of these to lactose synthesis. Only B4GALT1 interacts with UDP-gal (Fig 3). Once UDP-gal binds the N-terminus of B4GALT1, the enzyme shifts its conformation from an inactive to active state, revealing the LALBA binding

site [55,74,92,93]. Fascinatingly, this binding of LALBA to B4GALT1 subsequently increases the preference of B4GALT1 for glucose by 1000-fold [55,74], where hydrogen bonding maintains glucose in the catalytic site. Within the resulting LS complex, subsite F is positioned close to the galactosyl acceptor subsite of B4GALT1 to establish favorable interactions for glucose. What remains unclear is how the interactions of LALBA with glucose are stabilized by B4GALT1, where it has been suggested that AC1 residues participate in stabilization together with subsite F [77,94]. Because only a monosaccharide-sized binding site becomes available, extended sugars like N-acetylglucosamine cannot bind B4GALT1 in the presence of LALBA [55,74,92]. The LS then transfers D-galactose (derived from UDP-galactose) to the OH-4 position of glucose to create lactose (Fig 3), after which lactose and LALBA dissociate and B4GALT1 returns to its inactive conformation [55,74,92].

The regulation of LS activity

There are a number of factors that regulate and specify LS activity. To start with, there likely is a degree of functional complementarity between LALBA and B4GALT1 for a given species. As an example, LALBA isolated from the platypus did not facilitate lactose synthesis when paired with bovine B4GALT1, where the concentration of platypus LALBA required for optimal lactose synthesis was 20-fold higher when it was paired with bovine B4GALT1 [21]. In addition, disruptions in the acid-base balance and the concentration of ions can inhibit LS activity. For example, hydrogen protons are a byproduct of glycosylation, where the $Ca^{2+/}Mn^{2+}$ ATPase 1, TMEM165, works as a hydrogen exchanger to deacidify the Golgi. In keeping with this critical function for TMEM165, its conditional deletion in the mammary glands decreased LS activity and milk lactose content by 36% [95]. Likewise, a high concentration of potassium inhibited LS activity *in vitro*, but only in the concentration range at which potassium was bound to LALBA [79].

The provision of various non-glucose precursors, analogs, or intermediates can also interfere with and/or inhibit LS activity. For example, glucose analogs such as 4-deoxy-D-xylo-hexose and 4-azido-4-deoxy-D-glucose suppressed the ability of LALBA to bind glucose, while N-

acetylglucosamine acted as non-competitive inhibitor by changing the conformation of B4GALT1 from an inactive to an active state [96], thereby preventing the binding of LALBA or UDP-galactose due to steric hindrance. Likewise, the presence of LALBA at extremely high concentrations leads to it binding B4GALT1 while it is still in its active state, creating a complex comprised of B4GALT1, UDP-galactose, Mn, and LALBA that then precludes glucose binding due to steric inhibition [74]. Finally, uridine triphosphate also inhibited LS activity, although the mechanism is not understood [59]. Combined, despite the massive amount of LS activity and turnover within MEC, there clearly is a parallel sensitivity to the biochemical microenvironment that has barely been unearthed at the physiological and molecular level, especially when considering the heterogeneity of LALBA expression within the gland we mentioned earlier.

Secretion of lactose

Once synthesized, large quantities of lactose are rapidly packaged into secretory vesicles (Fig 2) alongside other proteins and ions for export from MEC by exocytosis. While it is well-established that vesicles containing milk proteins, lactose, and water are formed in the *trans* Golgi, the way in which fragile and osmotically-active secretory vesicles are transported to the apical membrane of the MEC is not well-defined and warrants further investigation [97–99]. Generally speaking, MEC utilize a combination of microtubules and microfilaments to direct secretory vesicles from one organelle to another and toward the apical membrane [100,101]. In keeping with this mechanism, agents that inhibited microtubule function disrupted milk secretion in goats, rats, and guinea pigs also decreased lactose secretion as well as the accumulation of glucose, pyruvate, citrate, glycerol, and lactate in MEC [102–106]. Similar outcomes, including a 50% reduction in glucose uptake and a 50% reduction in lactose content and LS activity, occurred when microfilaments were inhibited in mammary explants from lactating guinea pigs [101,105]. Once secreted, LALBA can also dimerize with itself, creating a potential feedback inhibitor that can promote MEC apoptosis via inhibition of histone deacetylase activity [107]. Without doubt,

there is a great deal that remains to be learned about secretory vesicles in MEC, their transit, and the expulsion of their contents into the alveolar lumen.

THE PHYSIOLOGY AND TIMELINE OF LACTOSE SYNTHESIS

Having defined the biochemical pathway of lactose synthesis, our next objective is to review the temporal changes that occur physiologically as part of the upregulation and maintenance of lactose synthesis during lactation. Not only does this consideration provide further insight into the regulation of lactose synthesis, but it also affords important information that can improve the translation relevance of this pathway.

The onset of lactose synthesis during pregnancy and lactation

Lactose synthesis by MEC can be first detected in mid- to late gestation during a period of secretory differentiation (also known as lactogenesis I), which corresponds to an increase in the size and number of organelles in MEC, and a small but appreciable increase in milk protein gene expression [108,109]. During this period the secretions that have accumulated in the alveolar lumen can diffuse paracellularly between MEC into the blood prior to their excretion in urine [108], such that lactose can be detected in the urine of pregnant humans by the second trimester [110,111]. Others reported that the concentration of LALBA (8 ng/ml) in the plasma of pregnant humans was stable between 28 and 14 weeks prepartum, and increased to a peak of greater than 1 μg/ml at parturition [112].

Lactose synthesis increases rapidly during the subsequent acute phase of secretory activation (also known as lactogenesis II). The timing of periparturient secretory activation is associated with a rapid decrease in circulating progesterone, albeit timing varies by species [109,113]. Secretory activation in humans occurs postpartum as plasma progesterone levels decline rapidly following delivery of the placenta, whereas in other species such as pigs and rats, secretory activation occurs prepartum when plasma progesterone levels decrease [113,114]. Secretory activation also coincides with the sealing of tight junctions at the apical border between

MEC, concomitant with the dramatic increase in the transcription of genes in support of copious milk production [108,115]. As discussed in our subsequent review [32], these and other changes within MEC are directed by a general increase in circulating glucocorticoid, insulin, and prolactin levels, and a decline in circulating progesterone and estrogen, where these changes are species-specific [109,113].

The onset of lactose synthesis and secretory activation can be detected by a number of means. A clinical indicator in humans can be the self-report of breast/chest fullness, although a more sensitive and reliable biomarker is the rapid decrease in the Na/K ratio in milk [116,117]. Likewise, the level of lactose and/or LALBA in milk, urine, or plasma are also excellent biochemical indicators of secretory activation. For example, a decrease in lactose and LALBA in plasma and urine paralleled a drop in circulating progesterone in humans [118,119], while in cows, serum LALBA decreased to 140 ng/ml at L14 from a peak of 1000 ng/ml at parturition [112].

Much of the information regarding the onset of secretory activation was obtained by assaying LS activity in mammary tissue slices, either by measuring activity of the functional complex or the individual activity of LALBA or B4GALT1. In all these cases, enzyme activity was expressed as nmol of lactose produced per min per mg of particulate protein. Using this approach, LS activity in mammary tissue slices from cows increased by 1.4 units between L(-30) and 7 days prepartum L(-7), and by another 3 units by L(40). Concomitant with the increase in LS activity, LALBA concentration in bovine mammary tissue increased from undetectable levels at L(-30) to 82 and 178 μg/g per wet weight of tissue by L(-7) and L(7), respectively [120]. Likewise, activity of LS in mammary tissue of goats was detectable by day 120 of pregnancy (G120) even in the presence of high plasma progesterone levels [67,68]. Similarly, in rodents, 20-30% of the rise in LS activity in mammary tissue occurred by G20 [121–123]. Prior to G16-18, mice had limited LS activity (1-3 ng/h/mg wet weight), which then increased to 33 ng/h/mg wet weight between G19 and 8 h postpartum. The activity of the LS was highest in lactating mice from L(2) through L(6) at 142 ng/h/mg wet weight [124].

In recent decades the study of lactose synthesis onset has shifted to the transcriptomic analysis of genes within the pathway. One challenge that precludes a clear definition of the genetic regulation of the timing of this onset and its rate-limiting factors has been the lack of temporal standardization for transcriptomic and proteomic analyses. Consistent with the aforementioned relationship between the sealing of tight junctions and secretory activation, Lemay et al. suggested that the best practice was to cluster gene expression signatures relative to the milk Na/K ratio [116]. In the discussion of physiological processes that follows, we include insights into the transcriptomic changes that occur in the mammary gland during secretory activation across species. We should point out that in the studies described below, differential gene expression was rarely correlated with changes in milk composition or volume.

The timing of glucose uptake onset and its conversion to UDP-galactose

The uptake of glucose into the mammary epithelium is central to the initiation and maintenance of lactose synthesis. In dairy animals there is wide variation in the relative increase in GLUT1 gene and protein expression during secretory activation [47,49,125]. In humans, GLUT1 increased 1.4-fold between 6 h and L(7) in MEC, GLUT9 and GLUT10 increased 7 and 8-fold, respectively, by L(4) from 6 h postpartum [48]. In mice and rats, GLUT1 gene expression increased approximately 3-fold by L(2) relative to that in late gestation [126,127]. Even though these data confirm a well-established increase in GLUT1 gene and protein expression in MEC around secretory activation, there is no clear consensus as to the level of GLUT1 gene expression required for maximal lactose synthesis [30].

Interestingly, limited information also exists regarding the temporal expression of genes within the lactose synthesis pathway around the time of secretory activation, outside of for *GLUT1* and *LALBA*. In human milk fat globule membranes first collected 6 h postpartum and then every 12 h for 4 days as a source of MEC-derived RNA, expression of *HK1, HK2*, and *HK3* mRNA was decreased, while that for *PGM1, GALK1, GALK2, PGM2, UGP2, GALE, GALT*, and *SLC35A2* was increased by L(4) compared to baseline samples. While the greatest fold-change in gene

expression was recorded for *GALK2, UGP2*, and *PGM1–3* by L(4) (relative to 6 h postpartum), only the expression of *UGP2, PGM1*, and *SLC35A2* was correlated with milk lactose concentration [48]. Similarly, in another study of milk fat mRNA obtained from breastfeeding patients and stratified by milk Na/K ratio to define colostrum, transitional, or mature milk, the expression of *SLC2A9, GALK1, PGM1, UGP2, GALE,* and *SLC35A2* was increased in transitional milk compared to that in colostrum, whereas the expression of *GALT* and *HK1* was unaltered [116]. In contrast to the change in hexokinase expression recorded in human MEC, the expression of *HK1* in the MEC of sows increased after parturition and maintained that level throughout lactation, while *HK2* expression increased 2.5-fold within 12 h of parturition, then returned to levels recorded in pregnancy by the end of lactation [47]. The expression of *SLC35A2* increased 1.88-fold by L(14) and then decreased by L(21), while the protein expression of *SLC35A2* increased by L(4) and then plateaued [49]. In lactating rats, the *Hk1* gene was expressed in mammary tissue samples from both pregnant and lactating rats, whereas *Hk2* was expressed only during lactation after its expression increased 2.44-fold by L(1.5) [127,128].

As highlighted in this and the previous section regarding mammary hexoneogenesis, lactose synthesis has a high degree of plasticity, allowing MEC to up- or down-regulate various biochemical pathways to ensure that milk synthesis is optimal at all times. The regulation of genes required for the conversion of glucose to UDP-galactose, such as PGM1 and UGP2, should be further examined, given that in humans the expression of PGM1 and UGP2 was strongly associated with changes in milk lactose concentration.

Temporal changes in B4GALT1 gene and protein expression

There is no doubt that the expression of B4GALT1 is a parallel key driver for the onset of lactose synthesis. At the genetic level, mRNA for *B4GALT1* is expressed in most cell types across various tissues and has a long 5' UTR with an extensive secondary structure. Importantly, to support lactation MEC increase their transcription of a 3.9kb B4GALT1 mRNA variant that has a shortened 5'UTR and increased translational efficiency (Fig 2). It is this 3.9 kb *B4GALT1* mRNA

transcript that helps support the rapid increase in lactose production in the early postpartum period [129,130]. That said, there is discordant evidence as to whether the expression of B4GALT1 in MEC is upregulated in the first week postpartum across species. Specifically, whereas the expression of the B4GALT1 gene was increased in RNA isolated from the milk fat globule membrane of colostrum samples compared to mature human milk in one study, it did not change over time in another [48,116]. In the MEC of pigs, expression of *B4GALT1* increased 3.34-fold between L(-3) and L(0), then plateaued by L(2) [47,49].

Temporal changes in LALBA gene and protein expression

Not surprisingly, the expression of *LALBA* in the mammary gland increases dramatically during secretory activation so as to facilitate the rapid onset of lactose synthesis. Transcripts for *LALBA* mRNA were undetectable in pig mammary tissue prior to G(90) then increased 156-fold between L(-14) and L(-2) [46,130–132]. In dairy cows, LALBA protein was not detected in mammary tissue at L(-30) [120], while in nulliparous sheep, *LALBA* mRNA was first detected in mammary tissue at L(-14) when its expression in MEC was heterogeneous [69]. Similarly, LALBA was first detected in mammary tissue of goats around mid-pregnancy [68]. In pigs, the level of *LALBA* mRNA in mammary tissue increased 11.8-fold between L(-14) and L(-3), and by another 1.3-fold between L(-3) and L(1) [47,49]. Between L(-5) and L(10), the expression of *LALBA* in mammary tissue from cows did not change [125].

As mentioned earlier, there are various ways that LALBA levels can be monitored to track the onset of secretory activation. The level of LALBA in the plasma of pregnant humans varies between individuals and over the course of a pregnancy, with the average value being 35.4 ng/ml within a range of 0 to 600 ng/ml [134]. While LALBA excretion into human urine has not, to our knowledge, been measured, the excretion of lactose into urine begins to rise between weeks 10 and 20 of gestation [111]. The concentration of LALBA in serum in early gestating heifers did not exceed 5 ng/ml prior to day 160 prepartum, then rose to 23-30 ng/ml between days 120 and 60 prepartum. In dairy cows the concentration of LALBA in the plasma increased from 221 ng/ml on

L(-4) to 919 ng/ml on L(0), then declined to plateau at 463 ng/ml by L(2) [135]. Plasma levels of LALBA in goats began to rise 10-12 weeks prepartum concurrent with proliferation of the alveolar epithelium [68]. In pigs the level of LALBA in the blood increased rapidly between L(-7) and L(-2), coincident with an increase in plasma prolactin and a decrease in plasma progesterone [132,133]. The LALBA protein could not be detected in the serum of pregnant rats [136].

Once lactation is established in humans, dairy animals, and rodents, the expression of the LALBA gene and its protein becomes relatively constant. However, this assertion must be weighed against the previous discussion about the potential for considerable heterogeneity of LALBA expression within the lactating gland. The LALBA mRNA transcript was one of the most abundant in the milk fat globule membrane isolated from human milk on both L(0.5) and at L(42) [48], where the content of LALBA in human milk peaked at over 4.9 mg/ml in the first few days postpartum, then decreased to 3.4 mg/ml a month later [137]. At the global level, the concentration of LALBA in milk from lactating humans in the United States was higher than from those in eight other countries (~3.4 mg/ml versus 2.4 mg/ml). In Mexico, for example, the average concentration of LALBA in human milk was only 2.1 mg/ml [65]. Bovine milk contains 1.2-1.5 mg/ml LALBA, which represents approximately 50% of all the whey proteins [138]. Equine milk is more similar to human milk, having a LALBA concentration of 2.4 mg/ml that represents ~30% of all whey proteins [138]. There is also substantial variation in the content of LALBA in the milk from mares, which ranges from 0.63 mg/ml to 2.94 mg/ml, depending on the breed and study [139]. Murine milk contains only 0.9 mg/ml of LALBA [140], while in the milk of rats, LALBA content varied between 1.5 and 8.5 mg/ml depending on stage of lactation [141]. In the Tammar wallaby, the LALBA content of milk (2.1 mg/ml) remained constant over a 40-wk lactation, even though the lactose concentration varied widely due to the progressive increase in its degradation into glucose and galactose as lactation progresses [25].

CONCLUSION

The synthesis of lactose plays a critical role in directing the optimal growth and development of the young across nearly all mammalian species. At the broadest level, milk lactose content regulates milk osmolarity and overall milk volume and has important implications for water utilization across a drying planet. Lactose is also the building block for complex tri- and oligosaccharides that, until recently, could not be precisely analyzed. There is also a rising appreciation for the importance of lactose in human milk volume regulation, neonatal nutrition, and immune system development.

While the general biochemistry of lactose synthesis was elegantly defined within the last 50 years or so, a range of processes remain to be elucidated. The interaction between LALBA and B4GALT1 has been primarily studied *in vitro,* yet many questions remain as to how changes in the level of intracellular metabolites, including glucose, affect LS activity *in vivo*, whether that be in humans or dairy animals. Likewise, the function and regulation of the glycosylation of LALBA has yet to be determined. Similarly, the extrinsic and intrinsic factors that regulate *PGM, UGP2, GALE, GALT, SLC35A2* expression should be defined as these genes appear to be rate-limiting for lactose synthesis during secretory activation.

Clearly, there is also an ongoing need to better understand the regulatory strategies that fine-tune the synthesis of lactose at the level of the whole animal, the mammary gland, and MEC, which also varies across species. Even though plasma glucose is the main precursor for lactose, non-glucose precursors contribute up to 40% of carbon required to form lactose and have been largely overlooked. The role and regulation of these precursors warrants further investigation, particularly in the context of metabolic syndromes involving chronic inflammation and disrupted homeorhesis, such as ketosis, obesity, and diabetes mellitus. At the same time, questions remain as to how the regulation of lactose synthesis affects the production of tri- and oligosaccharides. We continue this theme of highlighting various extrinsic and intrinsic sources of regulation within the second review [32], where we present a range of opportunities to modulate milk composition through the regulation of lactose synthesis.

The cross-species diversity in lactose synthesis also underscores the importance of selecting the appropriate *in vivo* and/or *ex vivo* model for studying lactose production and its regulation. There is an ongoing need to develop a *bona fide in vivo* or *ex vivo* system to define the effect of ions, glucose, UDP-galactose, lactose, pH, and hormones on LS activity. The field also still also needs an authentic model of lactose secretion so as to allow the closer study of the mechanisms by which lactose is packaged, transported, and exported from MEC into milk. Taken together, a great deal remains to be understood about lactose, a component of milk that is all too often mistaken for being just a small and simple component of milk.

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FIGURE LEGENDS

Figure 1. The relationship between lactose and fat content in the mature milk of different species. More comprehensive graphical presentations of the association between milk lactose and fat concentration have been presented elsewhere [1]. Data for fat and lactose content in the milk of the human, cow, goat, mouse, rat, dog, minipig are presented as the mean of published ranges. Tammar wallaby: Fat (40 mg/ml) was measured at 26 weeks of lactation and lactose (39 mg/ml) between 13 and 34 weeks of lactation [25,142]; Florida manatee: Fat (190 mg/ml) and lactose (not detected) at 30 weeks and at 2 years of lactation [9]; Human: fat (28-44 mg/ml) and lactose (61-79 mg/ml) between 40 and 180 days postpartum [5]; Lemur: fat (18 mg/ml) and lactose (81 mg/ml) at 72 days postpartum [5]; Cow: fat (33-54 mg/ml) and lactose (44-56 mg/ml) during mid-lactation [6]; Horse: fat (12.1 mg/ml, range 50-200) and lactose (63.7 mg/ml, range 58-70) during mid-lactation [143]; Goat: fat (40 mg/ml) and lactose (32-50 mg/ml) during mid-lactation [6]; Mouse: fat (190-220 mg/ml) and lactose (24-28 mg/ml) in mature milk samples [7]; Rats: fat (140-159 mg/ml) and lactose (11-41 mg/ml) in mature milk samples [7]; Rabbit: fat (152 mg/ml) and lactose (18 mg/ml)) in mature milk samples [7]; Dog: fat (24-134 mg/ml) and lactose (29-40 mg/ml) in mature milk samples [7]; (mini)Pigs: fat (77-100 mg/ml)) and lactose (43-56 mg/ml)) in mature milk samples [7]; Subantarctic fur seal: fat (510 mg/ml) and lactose (not detected) in mid-lactation samples [12]; Polar bear: fat (278 mg/ml)) and carbohydrate (26 mg/ml)) in yearlings mid-lactation sample [144].

Figure 2. A schematic representation of the biochemical and cellular requirements for lactose synthesis. Glucose and non-glucose precursors are taken up by the mammary epithelial cell at its basolateral surface. Some glucose is shuttled directly to the Golgi while and other glucose and non-glucose precursors are converted to UDP-galactose through a series of enzymatic reactions. The 3.9 kilobase B4GALT1 mRNA is preferentially and abundantly transcribed and translated during lactation relative to the 4.1 kilobase B4GALT1 mRNA. Some LALBA is

glycosylated in the smooth endoplasmic reticulum. The lactose synthase complex is formed by B4GALT1 and LALBA in the Golgi, which then joins glucose and UDP-galactose to form lactose while the UMP moiety is recycled. Lactose, LALBA, and B4GALT1 within vesicles are secreted by exocytosis, and are guided and supported by microtubules and microfilaments. Aquaporin 3 (AQP3), α-lactalbumin (LALBA), β-1,4-galactosyltransferase-1 gene (B4GALT1), calcium (Ca), dihydroxyacetone phosphate (DAP), endoplasmic reticulum (ER), galactose (Gal), glucose (Glc), glucose transporter 1 (GLUT1), glycerol (glyc), glycerol kinase (GK), glyceraldehyde-3-phosphate dehydrogenase (G3PD), hexokinase (HK), kilobases (kb), manganese (Mn), messenger ribonucleic acid (mRNA), pentose phosphate pathway (PPP), phosphoglucomutase (PGM), UDP-glucose-pyrophosphorylase 2 (UGP2), phosphate (P), solute carrier family 35 A2 (SLC35A2), uridine diphosphate (UDP), uridyl monophosphate (UDP), UDP-glucose 4-epimerase (GALE)

Figure 3. A graphical representation of the biomolecular process of lactose synthesis. (1) First, B4GALT1 is resident in the Golgi in its inactive confirmation. (2) Then, UDP-gal binds the N-terminus of B4GALT1. The enzyme shifts its conformation from an inactive to an active state, revealing the LALBA binding site. (3) Next, LALBA can bind B4GALT1, increasing the preference of B4GALT1 for glucose by 1000-fold. (4) Lactose synthase transfers D-galactose (derived from UDP-gal) to the OH-4 position of glucose to create lactose. (5) Lactose and LALBA dissociate and B4GALT1 returns to its inactive conformation. Abbreviations: α-lactalbumin (*LALBA*), β-1,4-galactosyltransferase-1 gene (*B4GALT1),* galactose (Gal), glucose (Flc), uridine diphosphate (UDP)

FIGURE 1

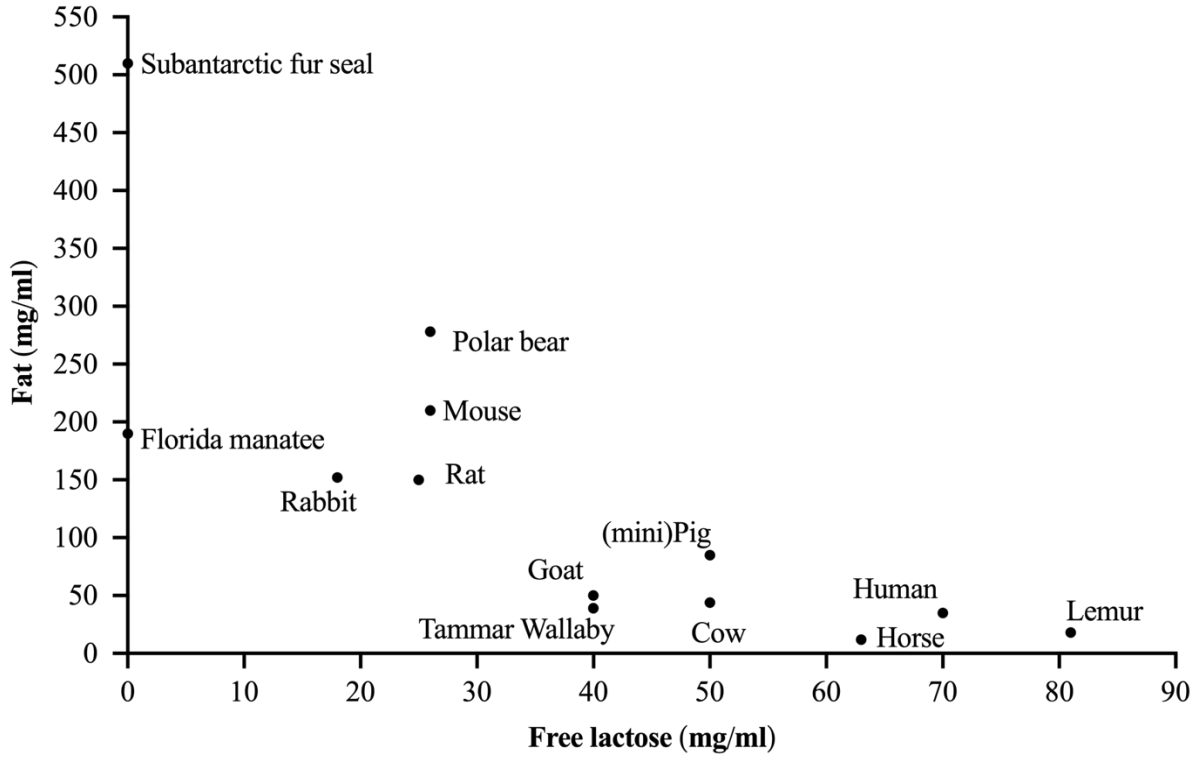

Fat (mg/ml)
550
500
450
400
350
300
250
200
150
100
50
0
Subantarctic fur seal
Polar bear
Mouse
Florida manatee
Rat
Rabbit
(mini)Pig
Goat
Human
Lemur
Tammar Wallaby
Cow
Horse
0
10
20
30
40
50
60
70
80
90
Free lactose (mg/ml)

FIGURE 2

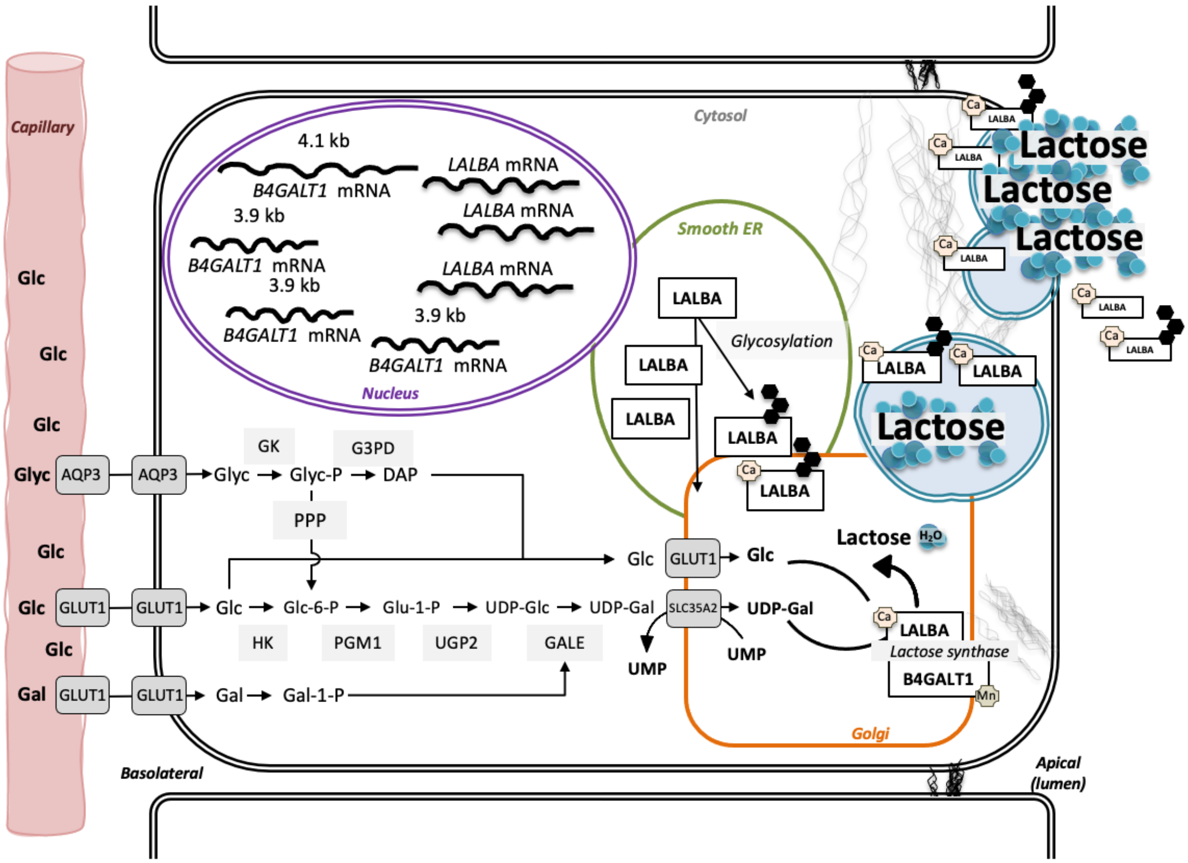
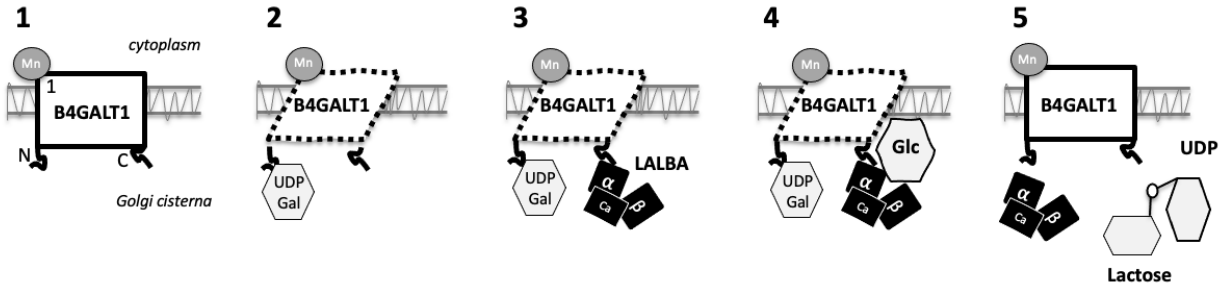

Capillary
Glc
Glc
Glc
Glyc
AQP3
AQP3
Glc
Glc
GLUT1
GLUT1
Glc
Gal
GLUT1
GLUT1
Cytosol
Nucleus
4.1 kb
B4GALT1 mRNA
3.9 kb
B4GALT1 mRNA
LALBA mRNA
LALBA mRNA
LALBA mRNA
Smooth ER
LALBA
Glycosylation
Glyc
Glyc-P
DAP
GK
G3PD
PPP
HK
PGM1
UGP2
GALE
Glc-6-P
Glu-1-P
UDP-Glc
UDP-Gal
SLC35A2
UDP-Gal
UMP
UMP
Gal-1-P
Lactose
Lactose synthase
B4GALT1
Ca
Mn
Golgi
Basolateral
Apical (lumen)

FIGURE 3


1
cytoplasm
Mn
1
B4GALT1
N
C
Golgi cisterna
2
Mn
B4GALT1
UDP
Gal
3
Mn
B4GALT1
LALBA
UDP
Gal
α
Ca
B
4
Mn
B4GALT1
Glc
UDP
Gal
α
Ca
B
5
Mn
B4GALT1
UDP
α
Ca
B
Lactose

Chapter 2. A comparative review of the extrinsic and intrinsic factors regulating lactose synthesis

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ABSTRACT

Milk is critical for the survival of all mammalian offspring, where its production by a mammary gland is also positively associated with its lactose concentration. A clearer understanding of the factors that regulate lactose synthesis stands to direct strategies for improving neonatal health while also highlighting opportunities to manipulate and improve milk production and composition. In this review we draw a cross-species comparison of the extra- and intramammary factors that regulate lactose synthesis, with a special focus on humans, dairy animals, and rodents. We outline the various factors known to influence lactose synthesis including diet, hormones, and substrate supply, as well as the intracellular molecular and genetic mechanisms. We also discuss the strengths and limitations of various *in vivo* and *in vitro* systems for the study of lactose synthesis, which remains an important research gap.

INTRODUCTION

The synthesis of lactose by the mammary epithelium occurs through a unique and conserved pathway that also varies across species. In a previous companion review [1], we outlined the extramammary, intramammary, and intracellular processes that direct lactose synthesis and secretion. The principal mechanisms involved in these processes include factors such as diet and hormones, and those specific to the transcription and post-translational modification of α-lactalbumin (LALBA). Not surprisingly, various species have evolved different approaches to regulate lactose production, which underscores the importance of selecting the appropriate model(s) for translational studies. In this manuscript we use a comparative, cross-species approach to review the key regulators and control points that modulate lactose synthesis and, in the process, outline the strengths and limitations of different *in vivo* and *ex vivo/in vitro* methods that have been used to generate these data.

BIPHASIC REGULATION OF LACTOSE SYNTHESIS BY PLASMA GLUCOSE LEVELS

Given our goal is to review the many different control points that regulate lactose synthesis, we start here by outlining the effect that glucose supply and availability can impart on the mammary epithelium. As described earlier [1], plasma glucose is the main precursor for lactose synthesis and plays a key role in determining milk volume. However, the effect of its availability on milk lactose yield or content is biphasic, as demonstrated through a range of studies in dairy cows using post-ruminal infusion of starch, glucose, or gluconeogenic precursors (i.e., casein) [2–5]. Of these, the most direct approach for studying the effect of plasma glucose on milk composition is close-arterial provision of different doses of glucose directly to the mammary gland [6].

At suboptimal plasma glucose levels, mammary blood flow becomes the primary driver of lactose production [5,7–10]. For example, when undernourished lactating goats were infused with

glucose, the yield and content of lactose in milk were highest when 50 or 60 g of glucose was infused per day. Specifically, when 60 g of glucose was infused, lactose content and yield increased to 48.1 mg/ml and 41 g from 46.4 mg/ml and 33.3 g, respectively, at baseline [6]. Mammary blood flow also increased in response to up to 60 g/d of exogenous glucose, then remained stable at levels of 80 or 100 g/d, while milk lactose content and yield decreased to baseline values (46.5 mg/ml and 35.1 g, respectively) at the 100 g/d dose [6]. Importantly, the level of glucose extracted by the mammary epithelium was constant across all doses. Likewise, the milk fat and protein content was not affected by the glucose dose [6]. During these states of adequate glucose availability there was also a parallel decrease in the level of glucose-6-phosphate in the mammary tissue and/or milk, as occurs in both rats [11,12] and goats [6].

On the other hand, during states of excess glucose supply there is a shift toward the intracellular accumulation of glucose metabolites that can suppress lactose synthesis. When 80 g/d glucose was infused to the udder, goats transitioned from negative to positive energy balance, and a larger amount of glucose left the gland unused [6]. A parallel indication of this shuttling of glucose away from lactose synthesis is the accumulation of glucose-6-phosphate in the mammary epithelium or milk. In dairy cows receiving excess glucose via post-ruminal infusion, the concentration of glucose-6-phosphate in milk increased while that of glucose-1-phosphate decreased [5]. These changes may reflect the actions of insulin responding to increased plasma glucose levels, where insulin is a strong negative regulator of phosphofructokinase, which would lead to an increase in glucose-6-phosphate levels [6,13].

The role of plasma glucose concentration and supply in the regulation of lactose synthesis in lactating humans remains less clear. Certainly the negative effect of hypoglycemia on breast milk lactose and yield in the setting of a prolonged fast is well-established [14–16]. However, the question of how excess plasma glucose modulates lactose synthesis still requires investigation. Whereas Neville et al. concluded that the elevation of plasma glucose to 8 mmol/l for 4-6 hours did not impact milk lactose content or the rate of lactose synthesis [17], this level of plasma

glucose is within normal limits for postprandial glucose levels. In a subsequent smaller experiment with three breastfeeding humans producing less than 500 ml daily, elevation of plasma glucose levels to 8 mmol/l resulted in a numerical increase in the lactose concentration in milk, from 189 to 203 mmol/l [17]. In our view, these findings warrant further validation in a well-powered study to clarify how varying plasma glucose levels impact milk lactose yield or content in humans.

In summary, it appears there is a biphasic effect of plasma glucose levels on lactose synthesis across ruminant and non-ruminant species. It must be noted that the physiology, lactose precursor requirements, evolutionary adaptations, and milk composition of ruminants and non-ruminants differ, and specific conclusions regarding the mechanism in one species cannot be attributed to that of another. It is tempting to speculate that at suboptimal plasma glucose levels, mammary blood flow is the predominant player in the regulation of lactose synthesis, while at excess plasma glucose levels, the accumulation of intracellular intermediates in the MEC contributes to a downregulation of lactose synthesis. It remains to be determined how, at the genetic, biochemical, and cellular level, this occurs and whether insights into this mechanism can be harnessed for tailored interventions to improve outcomes for those with metabolic dysregulation (i.e., diabetes mellitus or ketosis).

THE HORMONAL REGULATION OF LALBA AND B4GALT1 SYNTHESIS

As we discussed previously [1], the abundance of LALBA and B4GALT1 are key determinants for lactose synthesis, where their expression in the mammary epithelium is tightly regulated by critical hormones, including prolactin (PRL), glucocorticoid (GC), insulin (INS), triiodothyroinine (T3) and epidermal growth factor (EGF). Here we outline how these factors individually, or combined, can alter LALBA and B4GALT1 synthesis, with a prefaced overview of different *in vitro* systems that have been used to draw these conclusions.

In vitro models for studying LALBA and B4GALT1

Despite their widespread adoption and utility, as well as their essential role in defining biological mechanisms, various culture models face the significant limitation that they do not faithfully recapitulate the extent of lactose synthesis and secretion that occurs *in vivo*. In many ways, this longstanding conclusion is unsurprising given that MEC within the gland must coordinately associate with other epithelial cells, stroma, and the vasculature to achieve complete functional differentiation. While some aspects of differentiation such as the formation of dome-like structures do occur in primary cultures of MEC, the primary milk proteins they synthesize are caseins such as ß-casein (*CSN2*), not LALBA, which emphasizes that these cultures are more representative of an early- to midpregnant state [18]. As pointed out by others, gene expression for β-casein and the appearance of cytoplasmic lipid droplets do not reflect secretory activation (also known as lactogenesis II) [19–21]. One exception to these limitations is a system that used a > 2-week lag in culture, which conferred hormonal sensitivity to primary MEC that went on to synthesize and secrete LALBA into the medium (~1-10 ng/ml/day) [22].

Among the different *ex vivo/in vitro* systems available, different lines of evidence support that explanted mammary tissue best-approximates the *in vivo* state. When mammary glands from midpregnant or pseudopregnant mice were dissociated to acinar fragments or diced into explants, MEC maintained cell-cell associations, their cuboidal shape, and synthesized LALBA and lactose [23–25]. However, the use of mammary explants as a model faces certain limitations. Within hours of exposure to PRL, INS, and GC, explants from midpregnant mice had a transcriptomic signature similar to that recorded during secretory differentiation *in vivo*. By contrast, prolonged stimulation by a hormonal combination that would normally accompany secretory activation *in vivo* resulted in a transcriptomic signature that was vastly different from that described in fresh mammary tissue isolated from mice during early lactation [26]. A parallel challenge is sustaining the synthesis of milk components in fresh mammary tissue from lactating animals for more than a few hours *in vitro.* While the rate of lactose synthesis and secretion under these conditions can be sustained

in the short-term, a decline in lactose synthesis thereafter likely reflects, at least in part, the high metabolic rate of MEC. For example, when mammary tissue was isolated from lactating guinea pigs, half of the lactose present in the tissue was released into the culture medium within 5 min. Following a washout phase, lactose secretion into the medium was then constant for up to 2.5 h, during which time the release of lactose was 2-3 mg/g tissue/h [27]. After 48 hours however, β-casein and *LALBA* mRNA and protein levels in mammary tissue and MEC decreased precipitously, even in the presence of PRL, INS, and GC [23,24,28].

Despite the limitations of these *in vitro* systems, they have certainly provided valuable insight into the hormonal regulation of LALBA and B4GALT1 gene and protein expression, as outlined in Fig 1. In considering this summary, one should separately appreciate that the regulation of *LALBA* and *B4GALT1* expression by hormones *in vivo* may well differ from that *in vitro* given the presence of a potential myriad of physiologic influences including blood flow, nutrient supply, and varying hydrostatic and osmotic pressures. Moreover, cultured mammary tissue responds differently to hormones depending on the reproductive state of the donor [29,30]. To assist the reader, we provide Supplementary File 1 that documents the culture conditions and outcome measures used in the literature that we describe hereafter.

Prolactin

There is a clear requirement for PRL during the initiation of lactose and LALBA synthesis in most mammals, an effect that is most pronounced during the preparation for secretory activation. For example, in humans, plasma PRL levels peaked immediately after parturition, then fell to 50 to 100 ng/ml unless stimulated by suckling or pumping [44]. This transient elevation of plasma PRL levels was associated with a 3.5-fold increase in *LALBA* mRNA in the milk fat globule membrane after 6 h [45]. In a range of species including non-pregnant humans and non-human primates, the synthesis of lactose alongside milk secretion can also be induced by exogenous estrogen (E) and progesterone (P) combined with a PRL-secretagogue [31–33]. Lactose synthesis was also induced in pseudopregnant mares and rabbits treated with a PRL-

secretagogue or recombinant PRL, respectively. By contrast, endogenous PRL was sufficient to induce lactose synthesis in pseudopregnant heifers and goats [34–37]. Exposure of pregnant gilts to the PRL-secretagogue domperidone in late pregnancy also tended to increase the abundance of *LALBA* mRNA in biopsied mammary tissue by day 2 postpartum [38].

The effect of PRL on lactose synthesis during established lactation is less pronounced. Domperidone administered to lactating dairy cows did not promote lactose synthesis [39,40], while milk lactose content was increased in lactating dogs treated with the PRL-secretagogue, metoclopramide, during the first week of lactation [41]. Both domperidone and metoclopramide are prescribed to increase milk production in humans although the quality of evidence supporting their effectiveness is low [42]. Administering recombinant PRL to lactating humans with PRL deficiency or to those pumping for their premature infants increased milk lactose content from 53 to 63 mg/ml and the concentration of neutral and acidic oligosaccharides doubled, without affecting milk fat or protein content [43].

The positive effect of PRL on lactose synthesis occurs primarily at the level of *LALBA* and *B4GALT1* transcription, where PRL first binds the PRL receptor to activate the interconnected Jak2/STAT5 and PI3K–Akt signaling pathways (Fig 1). Phosphorylated STAT5 then binds other transcription factors such as the GC receptor (GR) prior to recruitment to promoter and enhancer regions of milk protein genes [46–48]. The critical role of PRL-induced signaling in lactose synthesis is highlighted by the fact that lactating mice with a conditional deletion of STAT5 within their mammary glands had decreased expression of *LALBA*, but not *CSN2* [49].

It is worth highlighting that the stimulatory effect of PRL on LALBA synthesis occurs in concert with other lactogenic hormones, specifically GC, INS, and T3. The synthesis of ß-casein and lipid in mammary explants from midpregnant mice and rats required PRL [50,51]; whereas LALBA could also be synthesized by explants cultured in a medium supplemented with only INS and GC, its synthesis was delayed by 24 h in the absence of PRL [52]. Similarly, in multi-week cultures of MEC from virgin and pregnant rats, the combination of INS and GC also stimulated

LALBA synthesis, albeit to levels that were 18-fold lower than those achieved by the combination of INS, GC, and PRL [22]. Likewise, the synthesis of LALBA in mammary explants from pregnant pigs was induced by PRL alone in a dose-dependent manner, while maximal LALBA synthesis required the combination of PRL, INS and GC [53].

The concentration of PRL required for maximal induction of LALBA synthesis *in vitro* also depends on the dose of GC, an effect that seems to be species-specific. Maximal LALBA synthesis by mammary explants from midpregnant rats occurred in the presence of a low concentration of GC (10 ng/ml) added to medium containing INS along with supraphysiologic levels of PRL (5 µg/ml). By contrast, when explants were cultured with a higher concentration of GC and PRL, as found in rats during late pregnancy (40 ng/ml and 1 ug/ml, respectively), the synthesis of LALBA was similar in the presence or absence of PRL [54]. The induction of LALBA by PRL in mammary tissue from midpregnant rabbits was enhanced by supplemental INS, but not GC [55].

Not surprisingly, an additional determinant of the extent to which LALBA synthesis responds to PRL is the reproductive state of the animal. Explants from postmenopausal humans required supraphysiologic concentrations of PRL (20 µg/ml) to initiate lactose synthesis, whereas a lower concentration (2 µg/ml) of PRL was required for the same response by explants from premenopausal individuals [29]. Likewise, induction of lactose synthase (LS) activity in mammary explants from virgin mice required either more time, or supraphysiologic concentrations of INS, PRL, and GC, to reach a short-lived peak in activity relative to the times and concentrations required for explants from pregnant or parous mice. When PRL, GC, and INS were added to mammary explants from parous mice, the ED_{50} of these hormones that was required to induce lactose synthase (LS) activity was much lower than the ED_{50} for ß-casein, implying that parity conferred a lower threshold for hormone-induced activation of LS [56].

The *in vitro* sensitivity of B4GALT1 levels to PRL matches changes in the concentration of B4GALT1 and PRL around the onset of lactose synthesis *in vivo*, and consistently differs from

that for LALBA. When PRL was added to mammary explants from midpregnant mice and rats, lactose synthesis first increased after 4 to 8 h [54,57]. By contrast, LALBA activity in mammary explants from midpregnant mice increased after 18 h [57,58]. The activity of B4GALT1 and LS in explants from midpregnant mice reached a maximum after 3 d in culture, concomitant with the secretion of lactose into the medium, whereas LALBA activity continued to rise until it peaked on day 6 of culture, by which time the secretion of lactose had decreased to its nadir and the activity of B4GALT1 and LS was low [57,58]. In a similar way, PRL was only able to induce a constant, linear rate of LALBA production in mammary explants from midpregnant rabbits after a 1-2 day lag [55].

We should point out that this apparent asynchronous induction of B4GALT1 and LALBA by PRL in mammary explants from midpregnant mice should be interpreted with caution. Specifically, at a physiologic concentration of PRL (50 ng/ml), the upregulation of B4GALT1 and LALBA in response to PRL were comparable [59]. Furthermore, other experimental conditions, such as the GC concentration, could explain a 2-fold greater induction of B4GALT1 by PRL, given that a high GC dose (5 ug/ml) was later found to specifically inhibit LALBA synthesis [60].

In summary, while PRL clearly directs the upregulation and maintenance of lactose synthesis, there are undoubted species- and concentration-specific differences in how PRL regulates *LALBA* expression, as well as how it cooperates with other hormones such as INS and GC. Some of these mechanisms still lack resolution. Moreover, there are still gaps in our understanding of how downstream effectors of PRL signaling cascades, in concert with other hormone-regulated pathways, regulate the expression of *LALBA* and *B4GALT1* at the genomic level.

Thyroid hormone

The ability of thyroid hormones to regulate lactose and milk production has been the subject of inconsistent investigation over several decades. Oral or intranasal thyroid hormone releasing hormone (TRH) administered to breastfeeding individuals for 4 weeks postpartum

increased PRL secretion, milk production, and in some cases milk lactose content without a change in milk protein or fat content [61–64]. The greatest positive impact of TRH on milk production and lactose content was among those with insufficient milk production who received TRH in the first week postpartum [61–64]. Conversely, lactation failure can be an early clinical manifestation of both hyper- and hypothyroidism [65].

The effect of hypo- or hyperthyroidism on lactose content in other species is less clear. Administering thyroxine (T4) to lactating cows increased daily lactose yield by 25% and milk lactose content from 52 to 54 mg/ml [66,67]. Even though T4 is essential for the galactopoietic effects of PRL in mice [68], there is limited data to support whether exogenous T4 affects their milk lactose content. The induction of hypo- and hyperthyroidism during lactation variably affected lactose synthesis in rats [69,70], where hypothyroidism lowered the milk lactose concentration on day 15 of lactation (L(15)), but was without effect on L(1) or L(21) [71].

Combined lines of evidence suggest that the positive effects of triiodothyronine (T3) or T4 on lactose synthesis are species-specific and occur through a direct effect on LALBA transcription. For example, adding T3 or T4 to cultured primary MEC from virgin or midpregnant rats did not stimulate LALBA synthesis [22], whereas others recorded a clear stimulatory effect of T3 on LALBA and lactose synthesis in explants of mammary tissue from mice [69,70]. The level of *LALBA* mRNA and protein increased 2-fold in mammary tissue from midpregnant mice in response to T3, whereas levels of mRNA for *B4GALT1,* CSN2 content, total RNA, and total protein synthesis were unaffected [72,73]. Whether T3 increased LALBA synthesis by exclusively stimulating transcription or extending the half-life of the *LALBA* mRNA transcript was not resolved [74].

One additional consideration is that T3 may modulate the actions of other hormones on MEC. Adding PRL to culture medium containing INS, GC, and T3 increased *LALBA* expression in murine explants by 40% above the level measured in cultures without T3 [72]. Whereas mammary tissue from virgin and midpregnant mice typically required supraphysiologic doses of

INS, GC, and PRL over three days to induce LS activity, supplemental T3 or T4 reduced the necessary dose of INS, GC, and PRL to physiological levels, and increased lactose synthesis 3-fold [56,72]. Over a range of concentrations, the L-forms of T3 and T4 were most stimulatory for LALBA synthesis, where the threshold for the induction of LALBA synthesis by L-T3 (10^{-10} M) was lower than for L-T4 (10^{-8} M) [72].

The regulation of lactose synthesis by thyroid hormones spans multiple levels and physiologic states and warrants continued investigation. In particular, the role of thyroid hormones for lactose synthesis is undoubtedly relevant for breastfeeding humans with thyroid disorders and clinical conditions involving metabolic dysregulation, such as obesity, as well as for high-producing dairy livestock that are prone to extreme negative energy balance. These questions also extend to the molecular level, where the action(s) of thyroid hormones on various milk protein genes, including LALBA, remain to be defined.

Progesterone (P)

The role for P during the initiation of lactation is clear, where its circulating levels must decrease to initiate the onset of copious lactose synthesis during secretory activation. This critical role for P is highlighted in postpartum humans with retained placental fragments, where secretory activation was delayed until the P-secreting placental tissue was removed [75,76]. The best demonstration of a mechanistic relationship between circulating P and the onset of secretory activation is the rapid induction of B4GALT1 and LALBA activity in mammary tissue homogenates isolated from rats following ovariectomy-induced depletion of P on day 19 of gestation [77]. This induction could be reversed when P was administered immediately after ovariectomy, whereas its inhibitory effect was less following administration 12 or 24 h later [77]. The effect of bilateral ovariectomy on total lactose content in mammary tissue was also evident in late-gestation rats 24 to 48 h after surgery, a response that was greater in rats ovariectomized later in gestation [78].

The mechanism by which P inhibits lactose synthesis primarily involves its repression of *LALBA* transcription. Notably, this repression is most pronounced in mammary tissue from

preparturient animals and is species-specific. For example, whereas P inhibited LALBA synthesis in mammary explants from virgin and early- to mid-pregnant mice, the same dose only inhibited LALBA production by 50% in mammary tissue from late-pregnant rats. In lactating rats the effect of P on LALBA synthesis was less, where a 1000-fold higher concentration of P was required to decrease LALBA content in mammary tissue from lactating versus non-lactating rats [30]. By contrast to these findings for rats, the P-induced suppression of *LALBA* in explants from lactating, non-pregnant cows was more sensitive than was *CSN2* or genes required for fat synthesis. For example, only 15 μM of P was required to inhibit *LALBA* transcription, whereas doses >30 μM were required to inhibit *CSN2* transcription [79].

Estrogens (E)

There are several indications that E can inhibit lactose synthesis during established lactation. Birth control pills delivering E+P decreased the content of LALBA in breast milk and overall milk production, although the volumetric decrease was still within the normal range of output [80]. Birth control pills containing E are also used to treat hyperlactation in humans, although the mechanism is undefined [81]. In lactating cows, a single dose of synthetic E accelerated mammary involution coincident with a reduced concentration of LALBA and lactose in milk following final milk removal [82,83]. In a similar way, a high concentration (30 μM) of 17-β-estradiol inhibited LALBA secretion by mammary explants from lactating cows by 35-45% [79]. When high doses of E were administered to goats during midlactation, they demonstrated a varied response in milk composition, with most having a progressive decline in milk yield. Among those goats, two animals had complete suppression of milk and lactose production within four days [84].

Beyond these responses, there is also evidence for a biphasic effect of E concentrations on LALBA and lactose synthesis. For example, a low dose (50 μg) of synthetic E increased milk production in ewes in late lactation whereas a high dose (5 mg) was inhibitory and decreased milk lactose content from 60 to 45 mg/ml [85]. In explants from midpregnant mice, low concentrations (1 or 5 ng/ml) of 17-β-estradiol, estrone, diethylstilbestrone, but not 17-α-estradiol, stimulated LS

and B4GALT1 activity, whereas a high concentration (5 μg/ml) of 17-β-estradiol was inhibitory for LS, but not B4GALT1, activity. The full effect of E on LS activity in these data was only evident 24 h after supplementation with T3 and physiologic levels of PRL or human placental lactogen. Even though the mechanism by which LALBA synthesis is stimulated or inhibited in response to E is unknown, the effect of E on LALBA synthesis in mammary explants was most apparent when the medium was also supplemented with a low, physiologic concentration of PRL [59,86].

Taken together, different lines of evidence support that E and P can modulate lactose synthesis during the onset of secretory activation and into established lactation. The inhibition of lactose synthesis through the P-induced downregulation of *LALBA* expression is most evident during pregnancy and in the hours immediately following the removal of the P-secreting tissue. Questions linger as to whether P remains inhibitory for lactose synthesis during lactation. The fact that different levels of E biphasically regulate *LALBA* expression is noteworthy and shares similarities with the biphasic response to different levels of GC we outline below. The relationship between E, T3, PRL, and P in the regulation of *LALBA* described thus far underscores the importance of developing *bona fide ex vivo* and *in vivo* systems for the study of lactose synthesis and milk production.

Glucocorticoids (GC)

The increasing secretion of cortisol by the adrenal glands during gestation prepares MEC for the onset of copious milk secretion. In fact, GC facilitate an array of cytological changes in MEC including the synthesis of rough endoplasmic reticulum, tight junction closure, increased PRLR expression, and regulation of milk protein gene expression [87]. In these ways, mammary explants from midpregnant mice entered a secretory state when exposed to hydrocortisone, corticosterone, or aldosterone at 1 or 5 μg/ml, while deoxycorticosterone was ineffective [88]. The effects of GC are also clearly evident when they are administered to pregnant animals, which invokes secretory activation with or without premature parturition, depending on the species [89–93]. As a case in point, milk lactose concentration and udder distension were increased in

multigravid goats within 24 h of a second dose of adrenocorticotropic hormone. Thereafter, the milk lactose content during the rest of the pregnancy did not return to pre-treatment levels, but instead remained elevated at levels seen in mature milk [93]. In a similar way, administering GC to pregnant humans induced secretory activation despite the high circulating level of P, as was evidenced by breast engorgement and increased excretion of urinary lactose [92,94]. Exogenous GC had less of an effect on the induction of lactose synthesis in humans further along in their pregnancy [91]. Interestingly, ewes that underwent precocious secretory activation in response to exogenous GC subsequently produced less milk with a lower lactose content [90,91].

There is also a clear impact of GC on milk production during established lactation. Such a relationship is most clear for plasma cortisol, which is negatively-associated with milk lactose concentration. However, the association between the concentration of cortisol in milk and its lactose content is less consistent [95–97]. This effect of GC on lactose synthesis, including the effect of exogenous GC, can be revealed in different models and states. For example, lactating humans who received an injection of exogenous GC for musculoskeletal pain had complete or near complete suppression of milk production within one day [98,99]. In the same way, synthetic GC administered to lactating cows reduced milk lactose concentration from 46 to 43 mg/ml within 24 h of treatment, concomitant with a decrease in milk yield of approximately 10 kg/d [100]. Rat and mouse pups whose dams received daily injections of cortisone had retarded growth within 24 h of treatment [101,102]. Likewise, hydrocortisone administered to rat dams for the first 15 d of lactation decreased total protein and lactose concentration in milk [103].

Another example of how GC potentially modify lactose synthesis can be recorded during times of stress, where both lactose and milk production decrease in association with a dysregulated hypothalamic-pituitary-adrenal axis and increased cortisol secretion [104]. Within 46 h of exposing lactating ewes to a stressful event, lactose content and milk yield decreased, whereas milk fat and protein concentration increased [105]. Similar responses were recorded in dairy cows exposed to transportation stress [106]. Intriguingly, goats did not demonstrate a

decrease in lactose synthesis or milk yield following exposure to a stressful event [107–109]. From these data across a range of species it is clear that endogenous GC and high doses of exogenous GC can negatively impact milk production and lactose synthesis.

A primary mechanism underlying the negative effect of GC on milk output likely involves the suppression of LALBA synthesis (Fig 1), an effect that varies depending on the developmental and lactational stage of the animal as well as the concentration and type of GC. Notably, GC exerted a differential effect on the expression of *LALBA* versus *CSN2* and *B4GALT1*, where low concentrations of GC stimulated *LALBA* expression in explants from midpregnant rats and mice, while high concentrations suppressed *LALBA* synthesis [24,110,111]. By contrast, the synthesis of B4GALT1 and CSN2 increased in response to GC in a dose-dependent manner [112,113]. As a case in point, maximal CSN2 synthesis occurred in response to hydrocortisone concentrations that were 200 times greater than those required for maximal LALBA synthesis [110,112]. Lactose synthesis within mammary organoids from mice also responded to increasing concentrations of GC in a biphasic manner [24,114]. For mammary organoids from lactating mice cultured on floating collagen gels, a low concentration of cortisol (0.03 μM) was more stimulatory for LALBA synthesis than a high concentration (3 μM) [115,116]. Similarly, in mammary explants isolated from lactating cows, deoxycorticosterone at 30 μM inhibited LALBA secretion by 35-45% without affecting glucose uptake [79].

While most of these studies were conducted using tissue or cells from mice, we should point out that other physiological factors likely impact the overall response to GC. For example, the biphasic effect of GC on LALBA synthesis in mammary explants isolated from virgin and midpregnant mice may well not exist for explants isolated from lactating mice [28,30,79,117–119]. Furthermore, the biphasic dose response by LALBA to GC was not observed in a long-term culture system using MEC isolated from either virgin or midpregnant rats [120]. Across these types of experiments there was also variation between individual animals in the amount of LALBA synthesized in response to low, stimulatory concentrations of GC when using mammary explants

isolated from late pregnant and lactating rats [28,30]. These types of variation likely reflect a combination of factors including heterogeneity within the mammary gland, as highlighted above, and interactions with other factors, as outlined below.

Not surprisingly, the effects of GC on LALBA synthesis *in vitro* are modulated by interactions with other factors including PRL, prostaglandins (PG), T3/T4, or spermidine. One such example is that the presence of GC decreases the dose of PRL required for maximal LALBA synthesis. Specifically, when an inhibitory high concentration of GC was added to cultures along with a lower concentration of PRL (0.5 μg/ml) and INS, the GC-induced suppression of LALBA synthesis was not as pronounced as it was in the presence of a higher concentration of PRL (5 μg/ml) [110]. A similar situation exists for PG, where it reversed the negative effect of high concentrations of GC on LALBA synthesis in cultures of mammary explants from midpregnant mice; the ED_{50} for PGE2, PGF2α, PGA2, and PGB2 to overcome the inhibitory effect of GC were 0.4, 0. 4, 10, and 10 μM, respectively [121]. Notably, PG could not stimulate LALBA synthesis after the GC-induced inhibition was reversed [122]. Unlike PG, thyroid hormone not only prevented the negative effect of a high GC concentration on LALBA synthesis, but also stimulated the synthesis of LALBA [122]. Lastly, the production of LALBA in mammary explants from midpregnant mice could be induced without GC when spermidine was added alongside PRL and INS at concentrations as low as 0.4 mM [123]. By contrast, the synthesis of LALBA by explants from midpregnant rabbits required only INS and PRL, but not spermidine or GC, whereas maximal LALBA synthesis in the explants from the midpregnant rat required the combination of INS, PRL, GC, and spermidine [124].

As outlined above, a role for GC in the regulation of milk synthesis has been dissected extensively *in vitro*, particularly with regards to its role as a co-regulator of milk protein synthesis. Surprisingly, the extent to which this hormonal modulation occurs *in vivo*, and the relevance of these findings to lactation and their potential role during environmental exposures such as stress and following the therapeutic use of GC in human and veterinary medicine, remains under-

investigated. Of particular relevance to these scenarios is the biphasic regulation of *LALBA* expression by GC, where its negative effect at high levels is likely through its direct effect on lactose and LALBA synthesis.

Insulin (INS)

Many *in vitro* studies have cemented the essential role of INS for *LALBA* expression at the level of the mammary epithelium, consistent with its widely recognized role in stimulating various milk protein genes (Fig 1) in concert with the effects of PRL and GC. For example, the expression of three genes involved in lactose synthesis, namely *LALBA, UGP2*, and *GLUT1*, increased in response to INS added to cultured mammary tissue from midpregnant mice [125]. Likewise, the expression of *LALBA* mRNA in mammary explants from late-pregnant cows increased 10-fold when INS was added to the culture medium [126].

Intriguingly, these robust effects of INS on lactose synthesis *in vitro* do not translate to a clear indication that plasma INS modulates lactose synthesis *in vivo.* This conclusion aligns with the widespread demonstration that glucose uptake by the mammary glands is INS-independent, consistent with the well-established fact that INS-dependent GLUT4 is absent in mammary tissue [127,128]. Infusion of INS also did not affect the arteriovenous difference for glucose across the mammary glands of goats, cows, or sheep. In a similar way, milk production and lactose synthesis by cows and sheep was unchanged in response to acute or chronic elevations of plasma INS during a glucose clamp experiment [129,130]. All these findings are consistent with the fact that a single dose of slow-release INS during the first week postpartum did not affect milk lactose output or milk yield from dairy cows [131].

These differences between the effects of INS on lactose synthesis *in vitro* and *in vivo* highlight how considerable gaps still remain in our understanding of both INS action and the regulation of lactose synthesis. Beyond the global role for INS in homeostasis and nutrient partitioning and its dysregulation across a range of conditions, there are still a number of questions that remain regarding its role in support of milk production.

β2-adrenergic receptor and its downstream effectors

It is also worth mentioning some of the early studies that examined the ability of signaling downstream of β2-adrenergic receptors to regulate lactose synthesis (Fig 1). Pregnant rats that received the β1- and β2- antagonist propranolol following the induction of secretory activation had a lower concentration of lactose in their mammary glands, whereas targeting the receptors pharmacologically using either prazosin (an α1 receptor antagonist) or metoprolol (a β1 receptor antagonist) had no effect [132]. By contrast, epinephrine and isoproterenol (β-adrenergic agonists) both inhibited the synthesis of lactose by cultured explants from lactating guinea pigs by 29% and 25%, respectively [27]. These opposing effects of β-adrenergic receptor signaling on lactose synthesis, albeit in two different species and in different physiological states, further highlights the need for a comparative approach to defining the control mechanisms underlying lactose synthesis.

The β2-adrenergic receptors are linked to the adenyl cyclase second messenger pathway (cAMP) and are regulated by PRL and ovarian hormones. The accumulation of LALBA within mammary explants from midpregnant mice decreased by 90% after supplementation with cAMP, whereas the ß-casein content decreased by only 35%. Sodium butyrate, 3'AMP, 5'AMP, ATP, ADP, and cyclic GMP did not affect LALBA synthesis. The inhibitory effect of cAMP on LALBA and ß-casein production was also augmented when a phosphodiesterase inhibitor was present [133]. In a similar way, lactose synthesis by explants from midpregnant mice and lactating guinea pigs was reduced following the supplementation of cultures with cAMP and phosphodiesterase inhibitors [27,134]. All these findings regarding the effects of β2-adrenergic receptor activation warrant further investigation given the importance of the neuroendocrine system in stress management and the widely-appreciated negative impact of stress on lactation performance.

Epidermal growth factor (EGF)

While EGF plays a crucial role as a paracrine growth factor in the developing mammary glands, there is also strong evidence to support it having a suppressive effect during the onset of lactation. In this way, LALBA activity in cultured explants from midpregnant mice was inhibited by 40% when they were exposed to EGF [135], similar to the suppressive effect of EGF on cultured MEC from lactating mice [116]. Similarly, synthesis of LALBA in ewes, rabbits, and mice was suppressed by EGF *in vivo* or *ex vivo*, where ewes in early lactation that received intravenous murine EGF produced less milk with lower lactose content [136]. Likewise, EGF suppressed the induction of *LALBA* by PRL in cultured mammary explants from midpregnant rabbits. Interestingly, this inhibitory effect of EGF was reversed by a low concentration of cortisol that also stimulated LALBA synthesis, whereas corticosterone and aldosterone reversed the suppressive effect of EGF, but were not stimulatory [51]. For reasons that are not entirely clear, the situation in rats appears different, where EGF promoted LALBA synthesis by cultured mammary explants from virgin and midpregnant rats [137]. In keeping with this positive effect, EGF also blocked the inhibition of LALBA synthesis by P in mammary tissue from pregnant rats [137].

Summary – hormonal regulation of LALBA and B4GALT1 synthesis

Taken together, it is perhaps not surprising that a milieu of hormones and their interactions can dramatically modulate lactose synthesis, which is achieved in a large part at the level of LALBA transcription. Many of these findings are based on some very detailed and thorough *in vitro* studies, particularly using mammary explants and relatively defined conditions. In our view, the physiological implications of these data are yet to be fully captured, whether that be for identifying ways to improve breastfeeding success, optimize milk production for dairy livestock, or support neonatal growth.

THE GENETIC REGULATION OF LACTOSE SYNTHESIS

In the previous section we detailed the impact of endocrine signals on lactose synthesis, particularly through their ability to positively or negatively affect *LALBA* expression. The nature of this regulation is, of course, particularly relevant during reproductive progression, as well as during adverse states such as stress. However, the synthesis of lactose is also determined at the genetic level, which applies across a range of taxonomic groups. Here we summarize a range of genetic mechanisms that directly regulate, or are associated with, altered lactose synthesis across numerous species and systems, with a primary focus on the genetic regulation of LALBA and B4GALT1.

Polymorphisms in genes outside the lactose synthesis pathway

The ability to screen for genetic polymorphisms in livestock including cattle, sheep, and horses has led to the identification of various genomic variants that are associated with measurable alterations in lactose output. In many cases, not surprisingly, these variants can be implicated in pathways underlying the synthesis of other major milk components including β-lactoglobulin [138–142], milk fat (1-acylglycerol-3-phosphate O-acyltransferase 6 and diacylglycerol O-Acyltransferase 1) [143–145], lactotransferrin [146] and the caseins [146]. In other cases, polymorphisms are more directly implicated in the hormonal regulation of the synthesis of lactose or other milk components, as is the case for the leptin receptor [143,147,148], growth hormone [146], growth hormone receptor, PRL, and suppressor of cytokine signaling 3 [138–142], or glucocorticoid receptor DNA-binding factor-1 [146] genes. While associative, these types of analyses can inform genetic selection strategies in livestock, where similar data accompanied by lactation performance measures will undoubtedly reveal a better understanding of the genetic regulation of lactose synthesis in humans.

Genetic variation in B4GALT1 and its impact on lactose synthesis

The B4GALT genes are expressed by most cell types to support intracellular glycosylation. By contrast, *B4GALT1* expression in MEC is tightly regulated during gestation and lactation to coordinate with, and support, lactose synthesis. Until mid-pregnancy, MEC transcribe

a 4.1 kb *B4GALT1* mRNA with a 175 nucleotide 5' untranslated region (5' UTR), concurrent with binding of Sp1 immediately upstream of a transcription start site (TSS). Subsequently, during late-pregnancy and throughout lactation, Sp1, CTF/NF1, and AP2 bind a different region, either ~200 bp upstream or downstream from the same TSS, yielding a truncated 3.9 kb mRNA transcript. This 3.9 kb mRNA transcript has a shorter 5'UTR that lacks an extensive secondary structure and has increased translational efficiency [1,149,150].

Several SNP exist within the bovine *B4GALT1* gene. Among nine SNP, three were associated with lower lactose content in milk whereas three others were associated with higher lactose content. Consistent with the aforementioned modulation of B4GALT1 mRNAs, one of these SNP was in the TSS and directed the switch between the long and short form of the *B4GALT1* 5'UTR in association with the milk having a lower lactose content. Two SNP were present in the B4GALT1 catalytic domain and were associated with a higher lactose content in milk. While SNP also exist within the region of B4GALT1 that interacts with LALBA, none were significantly associated with milk composition or volume [151].

Regulation of LALBA gene transcription

Given the critical role of lactose across a broad range of mammals, it is not surprising that the genetic structure of *LALBA* is widely-conserved, including its exon-intron boundaries [152–157]. The first three exons of *LALBA* are homologous to the lysozyme gene, while the fourth is unique [152]. In a similar way, a comparative analysis of the regulatory factor binding sites located in the *LALBA* promoter in the bovine, caprine, human, murine, rat, and swine genomes revealed three conserved motifs (LA1, LA2, LA3) located in the proximal end of the promoter sequences that were distinct from motifs found in the promoters of other milk protein genes [158].

The pronounced change in LALBA mRNA abundance during pregnancy and into lactation highlights how tightly its expression is coordinated at the transcriptional level. The murine *LALBA* proximal promoter (~2.5 kb upstream of the TSS) has an open chromatin structure across all reproductive states [159,160]. Surprisingly, the binding of only a few transcription factors to the

LALBA promoter has been assessed. The LALBA gene in rats and humans, as well as their five casein genes, all share an NF1 binding site in their proximal promoter [161]. The promoters for mouse, rat, human, and bovine *LALBA*, as well as the Ca-sensitive caseins and WAP, also have a conserved STAT5 binding site. Within the human *LALBA* promoter, these STAT5 binding sites are all proximal to steroid-hormone binding sites [162,163]. Additional repeated hexanucleotide sequences have also been identified in the human and rat *LALBA* promoter, although they do not resemble the consensus GC response element [153]. Consistent with this genomic landscape, both GR and pSTAT5 were bound to the murine *LALBA* promoter on days 1 and 10 of lactation [48]. Despite the fact that P clearly regulates lactose synthesis with the onset of lactation, it has not been established whether the *LALBA* promoter has a P receptor binding site in its 5'UTR [155]. Interestingly, the TATA, CCATT, GC response element boxes, and mammary gland-specific transcription factor sequences were not identified in a 500 bp region upstream of the Tammar wallaby *LALBA* coding sequence [164], perhaps reflecting the differential control of lactational output across developmental stage in this species.

In addition to regulation at the promoter, *LALBA* transcription is also influenced by its distal enhancer, which lies 1500 bp upstream of the bovine *LALBA* TSS [165]. This region is 75% homologous to the *CSN2* distal enhancer. While the *CSN2* distal enhancer has consensus binding sites for pSTAT5 and C/EBP, the transcription factors that bind the putative *LALBA* enhancer are yet to be defined [166,167], although GR and pSTAT5 were bound to the putative murine *LALBA* super-enhancer on L(1) and L(10) [48].

Superimposed on these transcriptional controls is an epigenetic landscape for the *LALBA* gene that is distinct from that for *CSN2* or *WAP*. In mice, the *LALBA* proximal promoter has an open chromatin structure across all reproductive states, which supports the notion that fine-tuning of *LALBA* transcription primarily occurs through the binding and tethering of transcription factor complexes to its proximal promoter [160]. The tailoring of an epigenetic environment in support of lactose synthesis is also illustrated by the fact that the different genes that contribute to lactose

synthesis all consistently maintain the chromatin modifications they acquired during pregnancy and lactation. By contrast, the epigenetic modifications surrounding the *CSN2* gene reverted to their pre-gestational state after involution [168].

Genomic variation and the regulation of LALBA function

The considerable genetic variation that exists within the LALBA gene across species also offers potential insights to its core functional elements. At the nucleotide level, there is a multitude of SNP within both the UTR and coding regions of the *LALBA* gene, although few have been analyzed for their association with milk yield or composition [165,169–177]. At one extreme, a single SNP 15 bp away from the *LALBA* TSS in Holstein cows was associated with higher lactose content and milk yield, but lower fat and protein content, and was proposed to account for a 30-fold greater expression of LALBA in explants from Holstein versus Angus cows [171,178,179]. Intriguingly, the same SNP in Swedish Red and White cows did not affect milk lactose concentration [178]. In a similar way, an I/V substitution at amino acid 46, the site of LALBA glycosylation, did not affect LALBA or lactose concentration in human milk [170], and none of four SNP in the 5'UTR of the equine *LALBA* mRNA was associated with altered LALBA mRNA or protein expression [177]. Among Chinese Holstein dairy cows, a T1847C SNP in a noncoding region was associated with lower lactose content and yield, but not fat or protein content [180]. Nine SNP were identified in the 5'UTR and 3'UTR of the Sarda goat *LALBA* mRNA transcripts, of which two SNP (-368 and -163) located at AP2α and SP1 transcription factor binding sites, respectively, were associated with lower milk lactose content [181].

What is perhaps even more enlightening is the genetic and associated phenotypic variation that exists within the LALBA gene across various marine mammals. The LALBA promoter in the Cape fur seal has a series of *cis*-acting mutations that results in the synthesis of a viscous, lactose-free milk with a high concentration of protein and fat [182]. In the California sea lion, the Antarctic fur seal, and the Cape fur seal, the *LALBA* TATA box has a T-G transversion (AAGAAA) in the third position that prevents binding of the TATA binding protein, thereby

preventing transcription initiation. However, the introduction of a STAT5 binding site and correction of the transversion in the TATA box in the LALBA promoter for the Cape fur seal did not activate gene transcription, suggesting that other mutations, like the disruption in the fourth exon found in the otariid LALBA gene, also contribute to the inability of the Cape Fur seal to synthesize LALBA and lactose [182,183]. Interestingly, the Atlantic walrus has a seven bp deletion that leads to a frame shift in exon 4 of *LALBA*, which translates to a longer 176 AA protein that is incapable of participating in lactose synthesis [183].

Taken together, these multiple layers of genomic and transcriptional regulation highlight how the genetic basis of lactose output has evolved as a tightly-coordinated program, while also being semi-independent from the expression of other milk proteins. There is also a great deal that remains to be learned about how these transcriptional controls are regulated and coordinated, not only across the lactational cycle, but also within individual cells and regions within the gland. Regardless, the combination of these insights points to a vast opportunity to harness and optimize these regulatory mechanisms, whether it be to manipulate milk composition or to improve the milk production potential in humans and livestock.

Post-translational control of LALBA

The LALBA mRNA and protein undergoes significant post-transcriptional and post-translational regulation and processing [184]. The primary site of LALBA glycosylation surrounds the N-glycosylation consensus sequence at Asn-45 [185,186], where glycosylation has been proposed to suppress the secretion of LALBA to allow for quality control at the level of the endoplasmic reticulum [187]. How the extent or nature of LALBA glycosylation impacts lactose synthesis and milk output is unclear, as we alluded to previously [1]. Introducing an Asn45Asp substitution into the water buffalo LALBA rendered it incapable of being glycosylated, although the associated milk composition was unchanged [186]. Goat LALBA contains two glycosylated residues at amino acids 45 and 74, yielding either an unglycosylated, singly- or doubly-glycosylated molecule [187]. Secretion of goat LALBA in a yeast culture system was suppressed

when the number of N-linked glycosylation sites was increased to three, whereas its secretion was highest when amino acid 45 was mutated and N-linked glycosylation was lost [187]. Certainly, there are physiological contexts where glycosylation of LALBA also varies. For example, adding EGF to explants from midpregnant rats cultured with INS, PRL, and GC decreased the synthesis of glycosylated LALBA by approximately 30%, such that the ratio of the two forms was 1:1 [188]. Conversely, supplementing cultures with thyroid hormone increased the abundance of glycosylated LALBA, whereas only non-glycosylated LALBA was produced by explants cultured in its absence [189].

Lessons from transgenic animals carrying an exogenous LALBA sequence

Transgenesis has served as a particularly innovative and insightful means to study and manipulate different aspects of the lactose synthesis pathway in animal models. We have elected to review those studies here, rather than in the respective sections above, because it is important to recognize that the context of situations like overexpression, heterologous systems, and altered physiological function can lead to different outcomes that may cloud any interpretations.

For some time a standing assumption was that the *LALBA* proximal promoter was sufficient to direct maximum gene expression, whereas optimal transcription of *CSN2* required its distal enhancer elements [165,190]. In early experiments, only short (<1 kb) *LALBA* promoter fragments were used to direct transgene expression in mice, based on the knowledge that many important, albeit undefined, *cis*-acting elements are located between positions -477 and -220 [191]. Transgenic mice with a longer 5' *LALBA* promoter fragment expressed bovine LALBA at approximately 1000 times higher concentrations than those harboring a shorter 5' fragment. While the resultant milk lactose content was not measured, transgenic mice that expressed higher quantities of bovine LALBA produced viscous milk [192]. When a 2kb *LALBA* promoter was used to direct the expression of bovine ß-casein in transgenic mice, the MEC underwent premature involution in association with more production of ß-casein and a viscous milk, similar to that described in *LALBA* knock out mice [193]. These findings contrasted with the phenotype of

transgenic mice expressing caprine ß-casein under the control of the caprine κ-casein promoter that maintained their milk production and composition. The authors proposed that the bovine LALBA 5'UTR sequestered transcription factors from the endogenous LALBA promoter, suppressing the production of LALBA and lactose [190,193,194].

Interestingly, a range of transgenic animal models has supported the general conclusion that overexpression of exogenous LALBA differently affects milk lactose content across species. Transgenic mice overexpressing human *LALBA* from a construct containing a 0.77 kb 5' fragment expressed the exogenous gene and protein at levels 14-fold greater than those for endogenous LALBA, without any effect on milk lactose content [195]. Transgenic sows bearing a bovine *LALBA* construct that included 2 kb of upstream sequence produced 20-50% more milk that had a higher milk lactose content and lower total solids, protein, and fat concentration than control animals [50]. This positive effect of bovine *LALBA* on milk composition was still apparent in the second lactation, where sows produced twice the amount of bovine LALBA in colostrum and milk versus during their first lactation [196,197]. The concentration of bovine LALBA in transgenic mice varied 10-fold between mice from the same transgenic line, suggesting that variation in the expression of exogenous LALBA was not just due to random integration of the transgene into the genome [192]. Likewise, the amount of human LALBA secreted into milk from transgenic cows varied from 0.17 to 1.56 mg/ml [198]. Transgenic cows only produced unglycosylated human LALBA, whereas transgenic mice produced bovine LALBA that was glycosylated at levels similar to those found in bovine milk [198,199]. Transgenic cows expressing human *LALBA* also expressed 43 unique proteins in the milk fat globule membrane without any apparent effect on the biology of milk synthesis [200].

These various animal experiments highlight the potential importance of regulatory elements within the 5' UTR of the *LALBA* gene. Combined with the aforementioned transcriptional regulatory mechanisms, it becomes clear that there is a host of conserved as well as species-specific regulatory elements that control and optimize *LALBA* transcription. These findings also

set the stage for future, more precise genetic modification strategies, such as those that can be edited using CRISPR/Cas9.

CONCLUSION

In this review we focused on defining the range of control points that regulate lactose synthesis, particularly at the endocrine and genetic levels. As we outlined above, combinations of intracellular and intramammary regulatory factors (Fig 1) are among the primary control points for lactose synthesis, more so than extramammary conditions like plasma glucose and blood flow. Nevertheless, dysregulation in the delivery of plasma glucose is inextricably tied to lactational output and is associated with stress and metabolic syndromes, such as obesity and diabetes mellitus. Plasma glucose availability and its uptake by the mammary gland for lactose synthesis is also modulated by the negative effect of fasting, caloric deprivation, and dietary carbohydrate restriction. Moving forward, one consideration is that lactating rodents may not be the best translational model for the study of food deprivation on lactose synthesis given their response is much more pronounced than that for lactating humans, and that they do not recapitulate the lower plasma glucose levels seen in lactating ruminants.

In considering the crucial role and regulation of lactose, there is no doubt that its synthesis and function(s) are a centerpiece for a range of emerging scientific concepts and global issues. Lactose plays a vital role in the movement of water which is a major component in dairy products worldwide. All these processes, as well as the survival of threatened species across a warming planet, depend on the movement of ever-scarcer water that is facilitated by the actions of lactose. At the same time, LALBA and lactose are critical for infant nutrition, as sources of protein and carbohydrate, respectively. Lactose also serves as the building block for a range of oligosaccharides that we now recognize have critical roles in regulating infant growth and development via the gastrointestinal microbiome.

With advances in genetic engineering and selection, there may also be ongoing opportunities to manipulate milk composition by targeting the lactose synthesis pathway. As a starting point, genetic mutations in the *LALBA* promoter that directly lead to a reduction in lactose synthesis need to be defined. Furthermore, the transcriptional regulators within the promoter and enhancer regions of the *LALBA* gene require better resolution as a way to screen and risk-stratify patients by their need for additional lactation support services or tailored therapeutic regimens. Many of these questions can now be pursued using mainstream sequencing technologies and non-invasive methods of studying the transcriptome from cells and the milk fat globule in milk. Special attention should also be placed on the species-specific effects of PRL, EGF, and thyroid hormone and the biphasic regulation of LALBA by E and GC, given that both steroids are involved in endogenous physiological responses and are common pharmacologic agents used in human and veterinary medicine. All these questions become additionally challenging to study given that there is an ongoing absence of *in vitro* models that mimic lactose synthesis and secretion, which hinders progress in the field. This issue of optimized models for milk synthesis *in vitro* becomes an important area for reconciliation that would have a significant translational impact across a range of applications.

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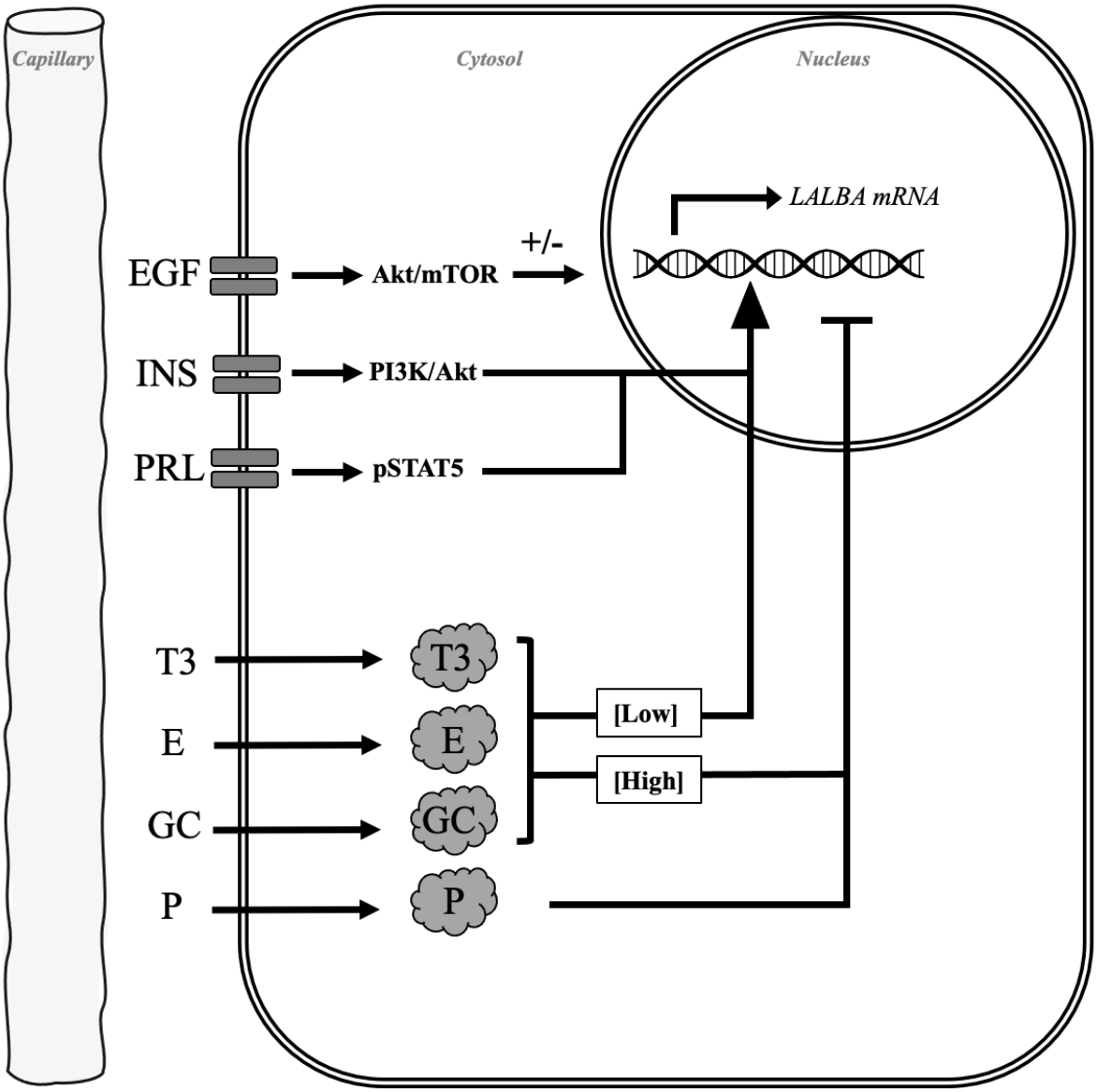
FIGURE LEGENDS

Figure 1. A schematic representation of the hormonal factors and mechanisms that regulate alpha-lactalbumin (LALBA) transcription. The positive regulators of LALBA transcription are prolactin (PRL) and insulin (INS). Progesterone (P) is a negative regulator of LALBA transcription. Thyroid hormone (T3), estrogen (E), glucocorticoids (GC), and epidermal growth factor (EGF) have variable effects on LALBA transcription that are species- or dose-dependent. Signaling occurs via intermediates including mammalian target of rapamycin (mTOR), phosphatidylinositol 3-kinase (PI3K), and phosphorylated signal transducer and activator of transcription 5 (pSTAT5).

Supplemental File 1. Culture conditions and outcome measures described in Chapter 2.

FIGURE 1


Capillary
Cytosol
Nucleus
LALBA mRNA
+/-
EGF
Akt/mTOR
INS
PI3K/Akt
PRL
pSTAT5
T3
E
GC
P
T3
E
GC
P
[Low]
[High]

SUPPLEMENTAL FILE 1

Species	Stage	Model	Culture conditions	Outcome measure	Author (Year)
Mouse	Virgin Midpregnant Parous	Explants	PRL: 10-2500 ng/ml INS: 1 µg/ml Cortisol: 1 µg/ml T3: 65 pg/ml	B4GALT1 activity in tissue LSC activity in tissue	Bolander (1983)
Mouse	Midpregnant	Explant	PRL: 0-1 µg/ml INS: 1 µg/ml Cortisol: 10-7 M	Lactose in tissue	Oppat (1988)
Mouse	Midpregnant	Explant	PRL: 0-1 µg/ml INS: 1 µg/ml Cortisol: 10-7 M	Lactose in tissue	Jagoda (1991)
Mouse	Midpregnant	Explant	PRL: 50 ng/ml INS: 5 µg/ml Cortisol: 5 µg/ml TH: 0.65 ng/ml	B4GALT1 activity in tissue LSC activity in tissue	Bolander (1980)
Mouse	Midpregnant	Explant	PRL: 1 µg/ml INS: 1 µg/ml Cortisol: 0.01-25 µg/ml T3: 65 pg /ml	B4GALT1 activity in tissue LSC activity in tissue Lactose in medium	Bolander (1981)
Mouse	Midpregnant	Explant	PRL: 5 µg/ml INS: 5 µg/ml Cortisol: 5 µg/ml HPL: 5µg/ml	B4GALT1 activity in tissue LSC activity in tissue	Turkington (1968)
Mouse	Midpregnant	Explant	PRL: 5 µg/ml INS: 5 µg/ml Cortisol: 3 µM PG: 0.03 - 3 µM	LALBA mRNA in tissue LALBA protein in tissue	Terada (1983)
Mouse	Midpregnant	Explant	PRL: 0.5-10 µg/ml INS: 5 µg/ml HC: 2.8 x 10-8 M	LALBA content in tissue	Ono (1981)
Mouse	Midpregnant	Explant	PRL: 5 µg/ml INS: 5 µg/ml Cortisol: 5 µg/ml TH: 0 - 10-6 M	B4GALT1 activity in tissue LSC activity in tissue Lactose in tissue	Vonderhaar (1975)
Mouse	Midpregnant	Explant	PRL: 1 µg/ml INS: 1 µg/ml	LALBA mRNA in tissue	Warner (1993)

			Cortisol: 50 ng/ml TH: 0.65 ng/ml		
Mouse	Midpregnant	Explant	PRL: 1 µg/ml INS: 5 µg/ml Cortisol: 0.1 µg/ml TH: 10-9 M	LALBA protein in tissue LSC activity in tissue B4GALT1 activity in tissue LALBA activity in tissue Lactose in tissue Lactose in medium	Bhattacharjee (1984)
Mouse	Midpregnant	Explant	PRL: 5 µg/ml INS: 5 µg/ml Cortisol: 0 - 1.0 µg/ml TH: 10-9 M	LALBA mRNA in tissue LALBA protein in tissue	Terada (1982)
Mouse	Midpregnant	Explant	PRL: 200 ng/ml INS: 100 ng/ml Cortisol: 50 ng/ml Fetal bovine serum	LALBA gene expression	Menzies (2009)
Mouse	Midpregnant	Explant	PRL: 5 µg/ml INS: 5 µg/ml Cortisol: 3 µM	LALBA protein in tissue	Perry (1980)
Mouse	Midpregnant	Explant	PRL: 1 µg/ml INS: 1 mg/ml Cortisol: 10-7 M	Lactose in tissue	Oppat (1989)
Mouse	Midpregnant	Explant	PRL: 5 µg/ml INS: 5 µg/ml Cortisol: 5 µg/ml Spermidine: 0.4 - 10-2 M	LALBA activity in tissue	Oka (1974)
Mouse	Lactating	Explant	PRL: 5 µg/ml INS: 5 µg/ml Cortisol: 0.01-1.0 µg/ml	LALBA mRNA in tissue LALBA protein in tissue	Perry (1984)
Mouse	Lactating	Organoid	PRL: 5 µg/ml INS: 5 µg/ml Cortisol: 0.01-1.0 µg/ml Fetal bovine serum	LALBA protein in tissue Lactose in tissue	Cline (1981)
Mouse	Lactating	Cell	PRL: 5 µg/ml INS: 5 µg/ml HC: 3 µM EGF: 50 ng/ml 10% FBS	LALBA protein cells LALBA protein in medium	Taketani (1983)

Mouse	Lactating	Primary cells	PRL: 5 µg/ml INS: 5 µg/ml Cortisol: 0.03 - 3 µM 5% FBS	LALBA protein in tissue LALBA protein in medium LALBA mRNA activity in tisue	Taketani (1986)
Rat	Virgin Midpregnant	Explant	PRL: 1 µg/ml INS: 0.1 µg/ml Cortisol: 1 µg/ml EGF: 0.01 µg/ml	LALBA activity in tissue B4GALT1 activity in tissue	Sankaran (1983)
Rat	Midpregnant Virgin (pseudopregnant)	Explant	PRL: 5 µg/ml INS: 5 µg/ml Cortisol: 1 µg/ml BSA: 2 mg/ml	Lactose activity in tissue LALBA activity in tissue B4GALT1 activity in tissue	Nicholas (1981)
Rat	Midpregnant Virgin (pseudopregnant)	Explant	PRL: 1 or 5 µg/ml INS: 5 µg/ml Cortisol: 0 - 1000 ng/ml P: 1 µg/ml	Lactose in tissue LALBA activity in tissue	Nicholas (1980)
Rat	Midpregnant	Explant	PRL: 1.0 µg/ml INS: 1.0 µg/ml HC: 0.05 µg/ml EGF: 0.02 µg/ml	LALBA activity in tissue Lactose in tissue	Sankaran (1988)
Rat	Pregnant Lactating	Explants	PRL: 1 µg/ml INS: 5 µg/ml RU26988: 3-300 nM	LALBA protein in tissue	Quirk (1988)
Rat	Virgin Midpregnant	Primary cells	PRL: 15 µg/ml INS: 15 µg/ml CORT: 0-50 µg/ml T3: 0.6 ng/ml 14% Fetal calf serum	LALBA protein in tissue LALBA protein in medium	Ray (1981)
Rat	Virgin Midpregnant	Primary cells	PRL: 15 µg/ml INS: 15 µg/ml CORT: 15 µg/ml T3: 0.6 ng/ml	LALBA protein in tissue LALBA protein in medium	Ray (1981)
Rabbit/Rat	Midpregnant	Explant	PRL: 5 µg/ml INS: 5 µg/ml Cortisol: 5 µg/ml Spermidine: 0.4 mM	B4GALT1 activity in tissue LSC activity in tissue	Bolander (1979)
Rabbit	Midpregnant	Explant	PRL: 1µg/ml INS: 0.1 µg/ml	LALBA activity in tissue	Sankaran (1984)

			Cortisol: 1µg/ml EGF: 0-1 µg/ml	LALBA activity in medium	
Pig	Midpregnant	Explant	Porcine/ovine PRL: 10 - 500 ng/ml INS: 5 µg/ml Corticosterone: 1µg/ml GH: 0.5 and 1.0 µg/ml T3: 0.1 and 1.0 ng/ml BSA: 1 g/l	LALBA protein in tissue LALBA protein in medium	Dodd (1994)
Cow	Lactating and nonpregnant	Explant	Bovine PRL: 0.5 mg/L INS: 1mg/L Cortisol: 1.4 µMol/L	LALBA protein in medium	Shamay (1987)

Chapter 3. Glucocorticoid-induced changes in gene expression within the udder of dairy cows and their association with reduced milk volume and changed composition

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ABSTRACT

Excessive glucocorticoids (GC) can transiently suppress milk output and milk lactose content without an effect on milk protein or fat concentration. The mechanism by which GCs suppress lactation has yet to be determined. We sought to define the effect of dexamethasone (DEX) on mammary gene expression in dairy cows to gain insight into the regulation of milk production by GCs. A single, high dose of DEX was administered to lactating Holstein cows and udder quarters were biopsied. Cows were quarter-milked before and after DEX and milk samples were analyzed for lactose, protein, fat, casein, solids, somatic cell count, α-lactalbumin (LALBA), and minerals. Total RNA was isolated from biopsied mammary tissue and subjected to RNA sequencing and differential gene expression analysis. Milk yield decreased concomitant with an increase in plasma glucose, a decrease in milk lactose and milk LALBA content, and no change in milk fat or protein content. The expression of the LALBA gene was transiently suppressed at 12 and 24 hours post-DEX. This work is the first to demonstrate a direct correlation between the administration of DEX and the concurrent suppression of milk yield, milk lactose and LALBA content, and LALBA gene expression. The extent to which a decrease in LALBA gene and protein expression contributes to the decline in milk lactose content and milk yield remains to be determined.

INTRODUCTION

Stress leads to decreased milk production in mammals [1–3]. Indeed, dairy cows that are stressed have a reduced responsiveness to oxytocin [4], depressed feed intake [5], and fatty liver [6]. The stress response is mediated in large part by glucocorticoids (GC), which have a range of physiologic properties, including immunosuppression and gluconeogenesis [7]. For this reason, synthetic glucocorticoids, such as dexamethasone (DEX), are the mainstay treatment for ketosis in veterinary medicine [8–10].

A parallel consequence of an acute, high dose of GCs is an abrupt, transient suppression of milk production [11–13], a response that is apparently more pronounced in humans and cows than goats [14]. When this suppressive response was monitored alongside arterio-venous differences in glucose uptake across the udder, Hartmann *et al.* identified that DEX had a clear negative effect on the ability of the udder to extract glucose from the circulation [15]. Further to these findings, Shamay *et al*. identified that during a DEX-challenge, the suppression of milk output was associated with a specific reduction in milk lactose output, while the level of milk protein and fat remained unaffected [11]. Given that lactose is the major osmole in milk [16], these findings point to a mechanism whereby a high level of DEX specifically suppresses the synthesis of lactose by the mammary epithelium. While GCs have also been implicated in regulating tight junction integrity [17,18], exogenous DEX had no effect on the ratio of Na/K in the milk of dairy cows [11], supporting a proposal that the effect of DEX on milk yield and composition was not via an effect on tight junction integrity.

Given that GCs can transiently suppress milk production, which may occur due to either a systemic effect or through local actions in the mammary gland, we sought to define the temporal transcriptomic response within the udder of dairy cows in response to a single administration of DEX. Our data reveal that a primary target of acute DEX exposure and its transient effect on milk synthesis is the lactose synthesis pathway, including the down-regulated transcription of α-lactalbumin (LALBA).

MATERIALS AND METHODS

Animals and Study Design

All animal experimentation was approved by the UC Davis Institutional Animal Care and Use Committee. Four non-pregnant Holstein cows (average 738.2 kg, range 648-838 kg) in their second lactation (average 55 DIM, range 40-64 DIM) were used. None had a prior history of clinical mastitis. Cows were maintained in separate pens and were bedded on rice hulls with *ad libitum* access to feed and water. Cows were fitted with rumination collars (SCR Engineers Limited, Israel).

The study period included an 8 day (d) acclimation period prior to the administration of a single injection of DEX. Four days prior to DEX, each cow was fitted with a jugular catheter that was flushed daily with saline and locked with heparinized saline (250 IU per ml). On day 9, each cow was administered a single injection of DEX (40 mg, 20 ml, IM, VetOne) between 07:00 and 09:30, immediately after the first biopsy and the subsequent milking. Blood was collected into vacutainers containing potassium oxalate and sodium fluoride every 12 hours (h) out to 5 d post-DEX and was processed by centrifugation at 2,000 x g for 10 minutes to yield serum that was stored at -80^{o}C until analysis.

Feed intake, composition, and rumination

Each cow was offered 20 kg of total mixed ration daily that was fed in equal amounts at 06:00 and 18:00. Refusals were collected and weighed at 18:00 for 5 d prior and 4 d following DEX. Feed (as-fed and refusals) was analyzed for moisture, dry matter, crude protein, adjusted crude protein, soluble protein, acid detergent fiber, neutral detergent fiber, lignin, nonstructural carbohydrate, starch, crude fat, and ash (Table 1) (DairyONE, Ithaca, NY).

Milk collection procedure and milk yield and composition analysis

Cows were milked twice daily with a portable milking machine to allow collection of milk from each quarter (QTR). The left rear QTR was designated as QTR1, the left front QTR was QTR2, the right front was QTR3, and the right rear was QTR4. During the experimental period,

milk was collected and weighed separately prior to (foremilk) and following (hindmilk) oxytocin (1.5 ml IV, 20 U/ml, VetOne, Boise Idaho). The fore- and hindmilk from each QTR was then combined and sampled in duplicate. A sample of hindmilk was also collected from QTR4. If a biopsy were to be performed, then milk was collected following the biopsy procedure to ensure there was no effect of milk removal on gene expression.

Duplicate milk samples were chilled on ice after supplementation with bronopol (Microtabs II, Nelson-Jameson) before storage at 4°C or -20°C. Refrigerated samples were analyzed for lactose, fat, casein, total protein, solids, and somatic cell count (SCC) (DairyOne, Ithaca, NY). Minerals (Na, K, Mg, Ca, Cl, and P) were analyzed in frozen milk samples (DairyOne, Ithaca, NY).

Milk α-lactalbumin

A bovine LALBA ELISA kit (Bethyl Laboratories, Montgomery, TX USA) was used according to the manufacturer's instructions to determine the content of LALBA in milk samples obtained at -24, -12, 0, 12, 24, 36, 48, 60, 72, 84 h relative to DEX. Concentrations of LALBA were established from a standard curve using bovine alpha-lactalbumin (RC10-128-6) provided by the manufacturer, where absorbance was measured at 280 nm. All samples were assayed in triplicate.

Serum glucose

Glucose levels were quantified using the Glucose Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Glucose concentrations were determined from a standard curve prepared with serially-diluted glucose, where absorbance was measured at 520 nm. All samples were assayed in triplicate.

Mammary biopsy

One or two cores of tissue (<100 mg per core) were collected from udder QTRs by needle biopsy (16g, Bard Magnum) at 0 (QTR1), 12 (QTR2), 24 (QTR3), and 72 h (QTR4) post-DEX to capture the anticipated full range in milk yield response [1]. Tissue was flash frozen in liquid nitrogen and stored at -80°C.

RNA isolation, cDNA library preparation, and sequencing

Total RNA was isolated from biopsied tissue (10-50 mg) from 4 cows at 0, 12, 24 h and 3 cows at 72 h using TRIzol (ThermoFisher Scientific) and 1-bromo-3-chloropropane according to manufacturer's instructions. The integrity of total RNA and yield were confirmed by formaldehyde gel electrophoresis and spectrophotometry (Nanodrop, ThermoScientific). Total RNA (5 µg) was then treated with DNase per the manufacturer's instructions (Zymo Research) and analyzed for quality (Experion™ RNA StdSens Analysis Kit), where all samples had an RNA integrity value greater than 8.3.

Gene expression profiling was performed using 3'Tag-RNA-Seq. Barcoded sequencing libraries were prepared using the QuantSeq FWD kit (Lexogen, Vienna, Austria) for multiplexed sequencing according to the manufacturer's recommendations, using 700 ng input RNA and 13 cycles of PCR for final library amplification. Fragment size distribution of the libraries was verified via micro-capillary gel electrophoresis on a Bioanalyzer 2100 (Agilent, Santa Clara, CA). The library masses were quantified on a Qubit fluorometer (LifeTechnologies, Carlsbad, CA), and pooled in equimolar ratios. The final pool was treated with exonuclease VII followed by bead clean-up to remove any free primer. The pool was quantified by qPCR with a Kapa Library Quant kit (Kapa Biosystems, loaction). Fifteen libraries were sequenced per lane on a HiSeq 4000 sequencer (Illumina, San Diego, CA) with single-end 90 bp reads generating an average of 6 million reads per sample.

Bioinformatic analyses

Raw reads were processed with HTStream (https://ibest.github.io/HTStream/) to remove adapter and low-quality sequences. On average, 0.2% of reads were removed. The trimmed reads were aligned to the *Bos taurus* UMD3.1 genome with Ensembl gene annotation release 93 using the aligner STAR v. 2.6.0c to generate raw counts per gene. On average, over 97% of the reads aligned to the *B. taurus* genome and 76% of the trimmed reads uniquely aligned to a *B. taurus* gene.

Prior to analysis, genes having an expression level less than 4 counts per million reads in all samples were filtered, leaving 10,241 genes. Differential expression analyses were conducted using the limma-voom Bioconductor pipeline (limma version 3.38.3, edgeR version 3.24.3, R version 3.5.1). The model used within limma was a single-factor ANOVA model for comparisons between timepoints and a linear regression model for correlations between continuous milk characteristics and gene expression. In all limma analyses, standard errors and estimates of log fold changes were adjusted for within-cow correlations. GO enrichment analyses were conducted by Kolmogorov-Smirnov tests as implemented in the Bioconductor package topGO (version 2.32.0.). Linear mixed effects models were used to evaluate the correlation between module eigengenes and the phenotype variables of total milk yield, total lactose %, total casein %, total protein %, total solids %, or total fat % and gene expression.

Genes that were differentially expressed at 12 and 24 h (adjusted $P < .05$) were filtered by up or down log-fold change and uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.8) using the *B. taurus* background list [19,20]. After selecting GOTERM_MF_DIRECT, the functional annotation chart was used where a threshold of two genes, an EASE score of 1, fold enrichment, and false discovery rate (FDR) were selected. Enrichment terms with an FDR greater than .05 were removed.

Gene lists for the lactose synthesis pathway and the gene ontology terms "Tight Junctions", “Inflammation”, “Response to Corticosteroids”, and “Regulation of Blood Vessel Diameter” [16,21] were used to align differentially-expressed genes at 12 and 24 h post-DEX. Finally, Enrichr (https://maayanlab.cloud/Enrichr/) was used to predict the upstream regulators in its Drug Signatures Database for differentially expressed genes at 12 and 24 h with an adjusted P-value < .05.

Statistical Analyses

Physiologic non-omic data were analyzed using GraphPad Prism (V 8.0). Milk yield and composition data were compared to an average value for the 0, -12, and -24 h timepoints relative

to DEX, unless otherwise stated. The effect of DEX on milk yield, composition, feed intake, rumination, or feed composition was analyzed using a two-way analysis of variance or Friedman's test, as appropriate, followed by multiple comparison testing. Significance was declared at $P < .05$.

RESULTS

Effect of DEX on rumination, feed intake, and plasma glucose levels

Rumination data were available for cows from 42 h prior to and 100 h after DEX. Whereas the rate of rumination for three cows was unaffected by DEX (Fig 1A), one cow (cow 1) developed hematochezia starting at 36 h post-DEX, ceased ruminating by 24 h post-DEX, and was removed from the study by 36 h post-DEX. Data for cow 1 are presented in Supplemental Figures. Dry matter-adjusted feed intake (Fig 1B) was not affected by DEX. Plasma glucose levels (Fig 1C) increased more than 2-fold by 12 h post-DEX ($P < .0001$), reached a peak of 167 mg/dl at 24 h, and returned to euglycemic values by 48 h.

Effect of DEX on milk yield and composition

Following DEX, average milk yield per 12 h interval decreased from 27.3 kg to 15.3 kg by 24 h, remained low at 36 h (19 kg, $P = .007$), and returned to baseline values by 60 h (Fig 2A). While energy-corrected milk transiently decreased from 31.3 to 22.6 kg by 24 h, this reduction was not significant (data not shown) [24]. One cow's milk yield decreased so much that no milk was collected prior to oxytocin, where the 24 h data point for that cow reflected milk only collected following oxytocin.

Milk composition data from -5 d to 96 h relative to DEX are presented for fore- and hindmilk from QTR4 (Fig 2 and 3). There was a tendency (main effect $P = .06$) for the lactose concentration in milk to decrease in response to DEX (Fig 2B), while the concentration of protein (Fig 2D) and casein (Fig 2E) increased (main effect $P < .05$). The concentration of LALBA in milk decreased

nearly 2-fold by 36 h (Fig 2H), where the main effect of DEX on milk LALBA content over time approached significance (P = .055). There was no change in the concentration of fat (Fig 2C), solids (Fig 2F), or SCC (Fig 2G) in milk throughout the experimental period. The concentration of lactose (P < .0001) in hind compared to foremilk samples was lower, while the corresponding fat (P < .001) and solids (P < .01) content was higher. There was no difference in the concentration of protein, casein, or SCC between fore- and hindmilk (Fig 2D, 2E, 2F).

The level of minerals and electrolytes in milk were assayed in QTR4 foremilk samples collected prior to oxytocin at 0, 12, 24, and 60 h relative to DEX. The concentration of Cl (Fig 3C, main effect P = .01) and Na (Fig 3D, main effect P = .04) decreased in response to DEX. The Na/K ratio (Fig 3F, main effect P = .04) decreased from 0.23 to a nadir of 0.19 at 24 h, then returned to the baseline value at 60 h. There was no change in the concentration of Ca, Mg, P, or K in milk in response to DEX.

Effect of DEX on mammary gland gene expression

Compared to baseline at 0 h there was differential expression of 519 and 320 genes in response to DEX at 12 and 24 h, respectively (Table 2). Importantly, no genes differed in their expression between the pre-DEX sampling at 0 h and at 72 h post-DEX (Table 2), highlighting complete reversal of the mammary gland transcriptome after exposure to DEX.

Using regression analysis, we identified seven genes whose expression had a log fold change that was negatively (RDH12, CEP57L1, SESN1, EPHX2) or positively (TUBA1B, AZGP1, TMEM35B) associated with a one-unit increase in milk yield (adjusted P < .05). By contrast, there were no genes having an expression profile that associated with the change in milk fat or lactose content. After adjusting for milk yield, the expression of one gene (ENSBTAG00000047609) was positively associated with a one-unit increase in milk fat content (adjusted P < .05).

We next characterized the specific changes in gene expression at 12 and 24 h after DEX. At 12 h, 519 genes were differentially expressed compared to baseline (0 h). Of these, the top ten most significant biological process ontologies (Table 3) were response to bacterium, immune

system process, actin cytoskeleton reorganization, positive regulation of fat cell differentiation, negative regulation of protein kinase activity, heart contraction, cGMP-mediated signaling, female gonad development, cellular response to cAMP, and response to drug. At 24 h, 320 genes were differentially expressed in response to DEX compared to baseline. For these, the top ten significant biological process ontologies were translation, cytoplasmic translation, formation of cytoplasmic translation initiation complex, intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator, response to oxidative stress, skeletal muscle tissue development, positive regulation of cell adhesion molecule production, proton transmembrane transport, negative regulation of proteolysis, regulation of apoptotic process. Of the 204 genes that had upregulated expression at 24 h in response to DEX, 136 were functionally annotated in the DAVID database. Twenty unique genes were significantly upregulated (FDR < .05) and belonged to three biological process ontologies: translation (RPL34, RPS27, RPS13, RPS2, EEF2, RPL13A, ENSBTAG00000047136, RPL5, MRPL10, RPL23A, RPL23, RPL24, RPL13, SLC25A3, RPL30), translational initiation (EIF2S3, EIF3E, EIF3D, EIF3H, EIF3F, EIF1), and formation of translation preinitiation complex (EIF2S3, EIF3E, EIF3D, EIF3H, EIF3F).

Effect of DEX on genes associated with inflammation, response to corticosteroid, blood vessel diameter maintenance, and tight junctions

Given the known relationship between GC exposure and global changes in gene expression [25], the local inflammatory response [26], blood flow [27,28], and tight junction integrity [17,18], we specifically examined DEX-induced changes in gene expression for these categories. In the category “Response to Corticosteroid” at 12 and 24 h, respectively, there were 10 and 5 genes having downregulated expression and 12 and 8 genes having upregulated expression (Fig 4). At 12 and 24 h post-DEX, respectively, there were 29 and 9 downregulated and 12 and 11 upregulated genes that were functionally categorized under the GO term “Inflammation” (Fig 5). Importantly, as described earlier, no cows had any sign of mastitis during the experimental period.

Several genes categorized under “blood vessel diameter maintenance” were differentially regulated in response to DEX, including 3 that were downregulated (ADD3, FGG, and SOD1) and 5 that were upregulated (CAV1, CBS, HMGCR, KCNMB4, KCNMB4, SNTA1) at 12 h post-DEX, of which 3 genes (CAV1, KCNMB4, SNTA1) remained upregulated at 24 h. There were 2 genes that were downregulated (CLDN15, ESAM) and 3 that were upregulated (USP53, C1QTNF5, YBX3) at 12 h post-DEX that were functionally classified under the GO term “Tight Junction,” while only 2 (DLG3, YBX3) genes remained upregulated by 24 h post-DEX.

Effect of DEX on genes in the lactose synthesis pathway

Genes in the lactose synthesis pathway were among those that were differentially expressed at 12 and 24 h post-DEX (Fig 6). Specifically, *LALBA* (Fig 6H), *AQP3* (Fig 6A), and *B4GALT1* (Fig 6B) were all downregulated (-1.1, -1.2, -0.7 log fold change, respectively, adjusted $P < .05$) at 12 h while the expression of *UGP2* (Fig 6I) was upregulated at this time (1.2 log fold change, adjusted $P < .05$). The expression of *LALBA* (Fig 6H) remained downregulated while *UGP2* (Fig 6I) was still upregulated (-1.3 and 0.8 log fold change, respectively, adjusted $P < .05$) at 24 h. The expression of *SLC2A1, GK, GAPDH, GALT, GALE, HK1, PGM1,* and *SLC35A2* was not affected by DEX. Notably, the expression of the β-casein gene (data not shown) did not change in response to DEX.

Predicted upstream regulators

Glucocorticoids were consistently among the top upstream regulators identified using the Drug Signatures Database in Enrichr, where flumetasone, diflorasone and fluorometholone were the top 3 predicted upstream regulators for the lists of genes that were differentially regulated by DEX at 12 and 24 h. The aryl hydrocarbon antagonist indolo[3,2-b]carbazole, the tyrosine kinase inhibitor mastinib (or AB1010), and the progestin analog etynodiol HL60 were the top three predicted upstream regulators of downregulated genes at 12 h while the chemotherapeutic valrubicin, the anthelminthic fenbendazole, and the antibiotic ofloxacin were predicted upstream regulators of downregulated genes at 24 h.

DISCUSSION

In this study, we extended the work of previous investigators by demonstrating that a single, high dose administration of DEX to multiparous dairy cows induced a transient decrease in milk yield and milk lactose and LALBA content concomitant with a suppression of *LALBA* gene expression. In line with previous findings [11,15], plasma glucose levels increased in parallel with a decline in milk yield within 12 h post-DEX. Even though LALBA mRNA and protein levels were also suppressed at 12 h post-DEX, lactose content did not decline until 24 h post-DEX. The decline in milk yield without a change in milk lactose, fat, or protein composition in the first 12 h suggests that GCs transiently suppress overall milk synthesis in a stepwise progression, whereby the initial response may be to limit glucose uptake by the mammary gland, without an increase in whole-body glucose production or utilization [12,15]. The subsequent negative effect of DEX on milk lactose content may have further suppressed milk yield. In the absence of data for mammary glucose uptake, it is not clear to what extent the decrease in mammary glucose uptake or the suppression of LALBA gene and protein expression contributed to the transient decline in milk lactose content.

Our findings implicate LALBA synthesis as being a key target of DEX in the udder of dairy cows. In rodents, the modulation of LALBA synthesis by GC is unlike that of other well-studied milk proteins such as β-casein and whey acidic protein. *In vitro* studies from the 1980s with mammary gland explants from midpregnant mice and rats first suggested that the GC-mediated regulation of LALBA synthesis may contribute to the pathophysiology of the stress-induced inhibition of lactation. The dose-response relationship between GC concentration and β-casein synthesis is classically sigmoidal and monotonic. By contrast, a low concentration of GC stimulated LALBA synthesis in mammary explants from midpregnant mice whereas a high concentration of GC suppressed it [29,30]. Our findings build upon this foundation by demonstrating for the first time that *LALBA*, but not β-casein gene expression, was transiently

suppressed in response to DEX *in vivo*. What remains to be established is the extent to which DEX-mediated suppression of *LALBA* gene expression contributes to milk production suppression.

The multifactorial role of LALBA offers clues into the evolutionary reason for the differential regulation of *LALBA* gene expression by GCs. In our work, *LALBA* gene expression was suppressed at 12 and 24 h and LALBA protein levels in the milk were lowest at 36 h post-DEX. Yet, milk lactose content returned to baseline levels by 36 h post-DEX following a nadir at 24 h. Even though milk lactose content is often proportional to milk volume due to its role as a primary osmole in milk, the relationship between LALBA and lactose content is not as straightforward. The LALBA protein is best known for its function in the lactose synthase complex within the Golgi of the mammary epithelium and, after its secretion into the milk, as a source of amino acids for the growing neonate. What is less appreciated is the role that LALBA plays in coordinating mammary involution. The accumulation of a dimeric form of LALBA (28 kDa) in the alveolar lumen during milk stasis results in the initiation of an apoptotic cascade in the mammary epithelium [31]. As a case in point, some aquatic animals can nurse their young once every few weeks without mammary involution because of a mutation in the *LALBA* gene which enables them to produce a milk devoid of LALBA and lactose [32]. It is tempting to speculate that in response to a stressful event, LALBA synthesis is downregulated such that less of it accumulates in the lumen and the mammary epithelium is protected from involution.

Inflammation appears to be central to the mechanism by which GC regulate milk production. It is well-established that mastitis, an inflammatory condition of the udder, in dairy cows is associated with a decrease in milk volume and milk lactose and LALBA content [33,34]. Interestingly, the gene expression of *LALBA,* but not β-casein or β-lactoglobulin*,* decreases in response to an infusion of lipopolysaccharide (LPS) into an udder quarter [35]. Recently, it was determined that an LPS infusion into the udder resulted in an decrease in milk fat and protein content, increase in milk SCC, decrease in plasma glucose levels, and an upregulation of genes

in the mammary epithelium that are regulated by GCs [36]. Not surprisingly, the effect of LPS on milk composition, plasma glucose, and mammary gene expression is in direct opposition to that of DEX, with a few key exceptions. The common denominator between our work and that of Shangraw *et al.* is the upregulation of genes downstream of activated GR and the downregulation of lactose synthesis. It appears that both a pro-inflammatory (i.e., LPS) and an anti-inflammatory (i.e., DEX) signal to the mammary gland can result in a downregulation of milk production via a decrease in lactose content, with GC-responsive genes, including LALBA, at the intersection of these experimental situations.

Limitations

A significant limitation of this study is the small sample size. One of the cows developed an adverse reaction to DEX and was removed from the study at 36 h post-DEX. Her physiologic and gene expression data were not included in the analyses and have instead been presented separately in Supplementary Figures. It is also important to note that milk samples from QTR1, QTR2, and QTR3 at 0, 12, and 24 h post-DEX, respectively, were bloody and could not be analyzed for composition. Similarly, QTR4 was biopsied at 72 h post-DEX which created milk samples thereafter that were bloody and unsuitable for analysis.

CONCLUSION

This work is an important step towards understanding how stress and exogenous GCs contribute to lactation suppression. We demonstrated for the first time that a single administration of a high dose of DEX to lactating dairy cows resulted in the transient suppression of *LALBA* expression concomitant with a decrease in milk LALBA and lactose content and milk yield.

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TABLES

TABLE 1

Table 1. Nutrient composition of total mixed ration

Nutrient Composition	
% of dry matter	
Crude protein	19.1
Adjusted crude protein	19.1
Soluble protein	26
Acid detergent fiber	19.4
Neutral detergent fiber	31.5
Lignin	5.95
Dry matter basis	35.4
Starch	19.45
Crude fat	5
Ash	9.01
Total digestible nutrients	68.5
Total nitrogen	3.06
Mcal/kg	
Net energy for lactation	1.61
Net energy for maintenance	1.61
Net energy for gain	1

TABLE 2

Table 2. Pairwise comparisons of differential gene expression in response to DEX

Comparison (h)	Number of Genes*
0 v 12	519
0 v 24	320
0 v 72	0
12 v 24	99
12 v 72	519
24 v 72	516

*Adjusted P < .05, n=3 cows
Abbreviations: hour (h), versus (v)

TABLE 3

Table 3. The top ten most significant biological process ontologies for genes that were upregulated or downregulated in response to DEX.

Name	Count	Up	Down
Response to bacterium	8	CAV1, COLEC12, IRAK1, TICAM2, LPO	CFD, MPEG1, TLR4
immune system process	36	TSPAN6, B4GALT1, CAV1, HSP90AB1, CD46, IMPDH2, CNOT7, COLEC12, STAT3, IRAK1, DDIT4, TICAM2, PHB, MPP1, LPO, GCNT1, FST, PTX3, SNX10	VAV1, TLR3, BLA-DQB, TMEM106A, ALOX15, PSMB9, ENPP3, CD320, LGALS9, CASP4, CFD, LGALS1, SOD1, AQP3, MSN, PSMB10, TLR4
actin cytoskeleton reorganization	1		PDLIM4
positive regulation of fat cell differentiation	1		MEDAG
negative regulation of protein kinase activity	7	CAV1, HMGCR, DNAJA1, GSKIP	FABP4, TRIB2, WARS1
heart contraction	3	SNTA1, CAV1	SOD1
cGMP-mediated signaling	1	PDE2A	
female gonad development	2	FST	SOD1
cellular response to cAMP	1	FDX1	
response to drug	4		FBP1, SOD1, PDE2A, SLC1A3

FIGURE LEGENDS

Figure 1. Effect of dexamethasone (DEX) rumination (1A), feed intake on a dry matter basis (1B), and plasma glucose (1C). Data are presented as mean +/- SEM for 3 cows. Time zero represents an average of -24 and 0 hours for feed intake and composition, an average of -24, -12, and 0 hours for plasma glucose, and an average of 12 data points from 0 to –24 hours for rumination. Only significant pairwise comparisons ($P < .05$) are shown. *, $P < .05$; **, $P < .01$.

Figure 2. Effect of dexamethasone (DEX) on milk yield (2A), lactose content (2B), fat content (2C), protein content (2D), casein content (2E), solids content (2F), somatic cell count (2G), and alpha-lactalbumin (LALBA, 2H). Data are presented as mean +/- SEM for 3 cows. Time zero represents an average of -36, -24, -12, and 0 hours relative to DEX for all components except for LALBA, where the baseline value is the average of -24, -12, and 0 hours. Only significant pairwise comparisons ($P < .05$) are shown. *, $P < .05$; **, $P < .01$; ***, $P < .001$; ****, $P < .0001$.

Figure 3. Change in content of milk calcium (3A), phosphorus (3B), chloride (3C), sodium (3D), potassium (3E), and sodium:potassium ratio (3F) in response to dexamethasone (DEX). Data are presented as mean +/- SEM for 3 cows. Time zero is one datapoint per cow (not an average of previous values). Only significant pairwise comparisons ($P < .05$) are shown. *, $P < .05$.

Figure 4. A heatmap depicting changes in differential gene expression in response to dexamethasone (DEX) of genes that are categorized under the gene ontology term “Response to corticosteroid.” Only genes with a significant (adjusted P-value < .05) change in expression and a log fold change between -2 and 2 in response to DEX are shown. Gene expression data for 3 cows are presented.

Figure 5. A heatmap depicting changes in differential gene expression in response to dexamethasone (DEX) of genes that are categorized under the gene ontology term “Inflammation.” Only genes with a significant (adjusted P-value < .05) change in expression and a log fold change between -4 and 4 in response to DEX are shown. Gene expression data for 3 cows are presented.

Figure 6. Differential gene expression in response to dexamethasone (DEX) for genes in the lactose synthesis pathway. Data are presented as mean +/- SEM for 3 cows. Letters a and b indicate significant differences between timepoints (adjusted $P < .05$).

Supplementary Figure 1. Effect of dexamethasone (DEX) rumination (A), feed intake on a dry matter basis (B), and plasma glucose (C) for Cow 1. Data are presented as the raw value at each time point.

Supplementary Figure 2. Effect of dexamethasone (DEX) on milk yield (A), lactose content (B), fat content (C), protein content (D), casein content (E), solids content (F), somatic cell count (G), and alpha-lactalbumin (LALBA, H) for Cow 1. Data are presented as the raw value at each time point.

Supplementary Figure 3. Change in content of milk calcium (3A), phosphorus (3B), chloride (3C), sodium (3D), potassium (3E), and sodium:potassium ratio (3F) in response to dexamethasone (DEX) for Cow 1. Data are presented as the raw value at each time point.

Supplementary Figure 4. Differential gene expression in response to dexamethasone (DEX) for genes in the lactose synthesis pathway for Cow 1. Data are presented as the normalized counts at each time point.

Supplementary Figure 5. Two heatmaps depicting changes in differential gene expression in response to dexamethasone (DEX) of genes that are categorized under the gene ontology term “Response to corticosteroid” (Left) and “Inflammation” (Right) for Cow 1. Only genes listed in Figures 4 and 5 for 3 cows are presented here for Cow 1.

FIGURES

FIGURE 1

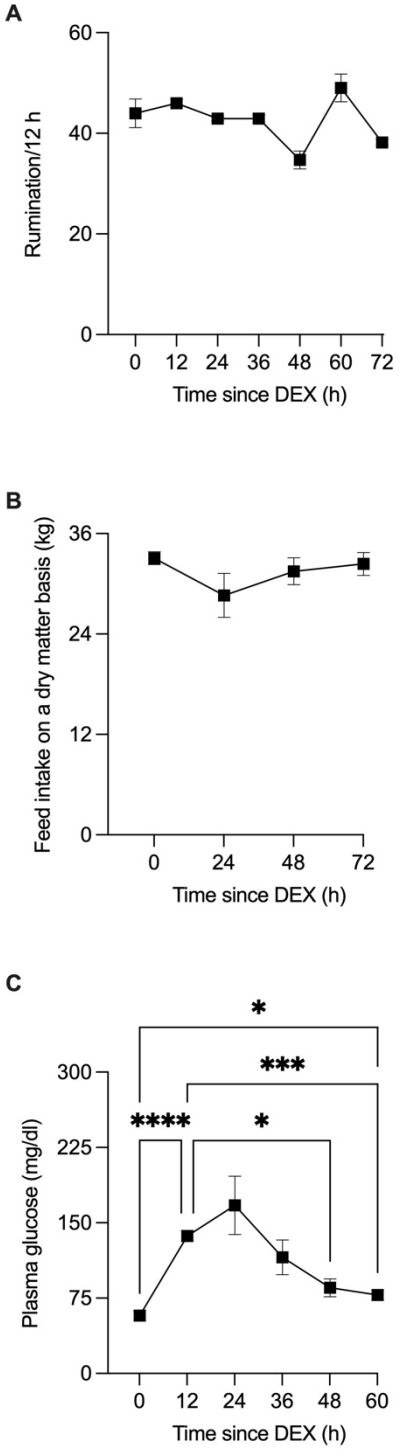


FIGURE 2

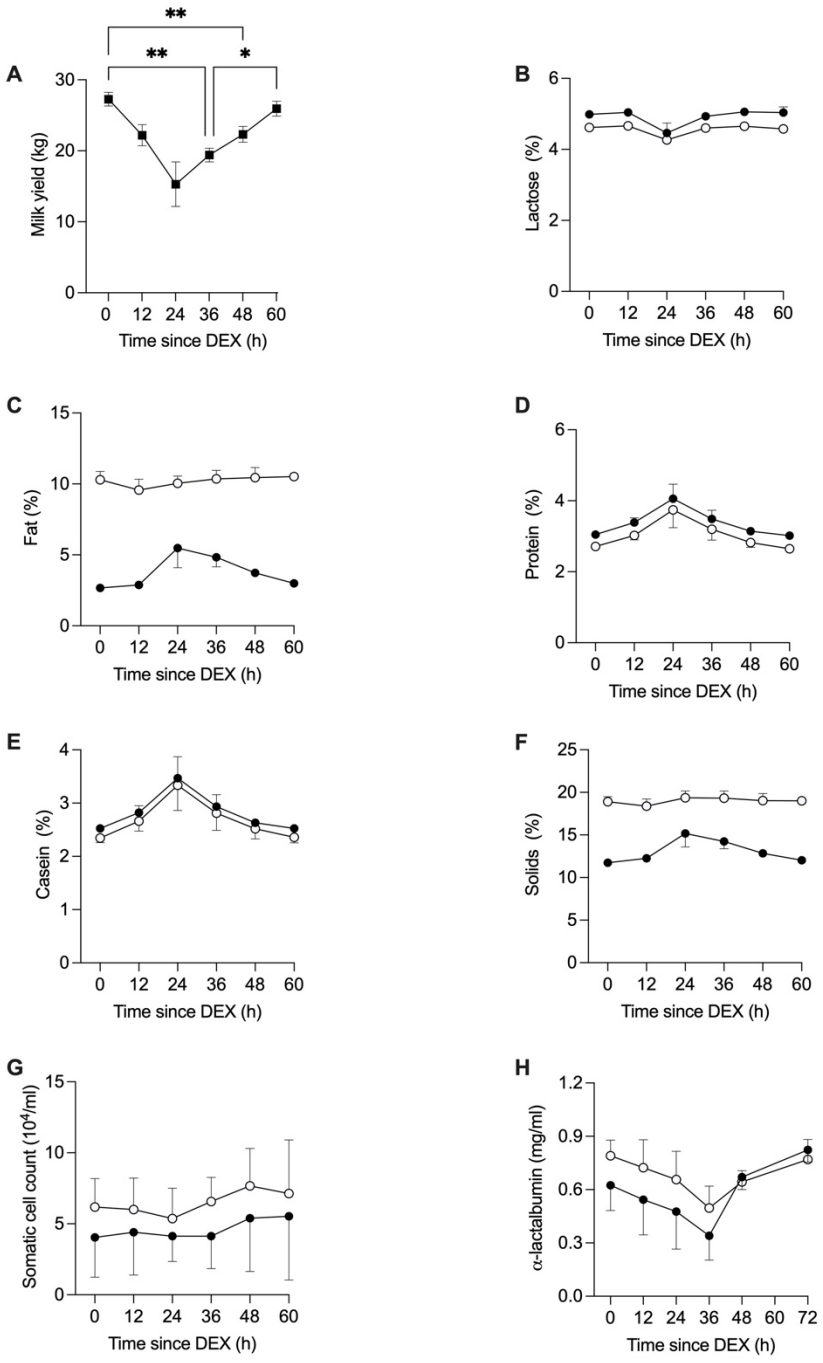

A
**
**
*
Milk yield (kg)
Time since DEX (h)
B
Lactose (%)
Time since DEX (h)
C
Fat (%)
Time since DEX (h)
D
Protein (%)
Time since DEX (h)
E
Casein (%)
Time since DEX (h)
F
Solids (%)
Time since DEX (h)
G
Somatic cell count (10³/ml)
Time since DEX (h)
H
α-lactalbumin (mg/ml)
Time since DEX (h)

FIGURE 3

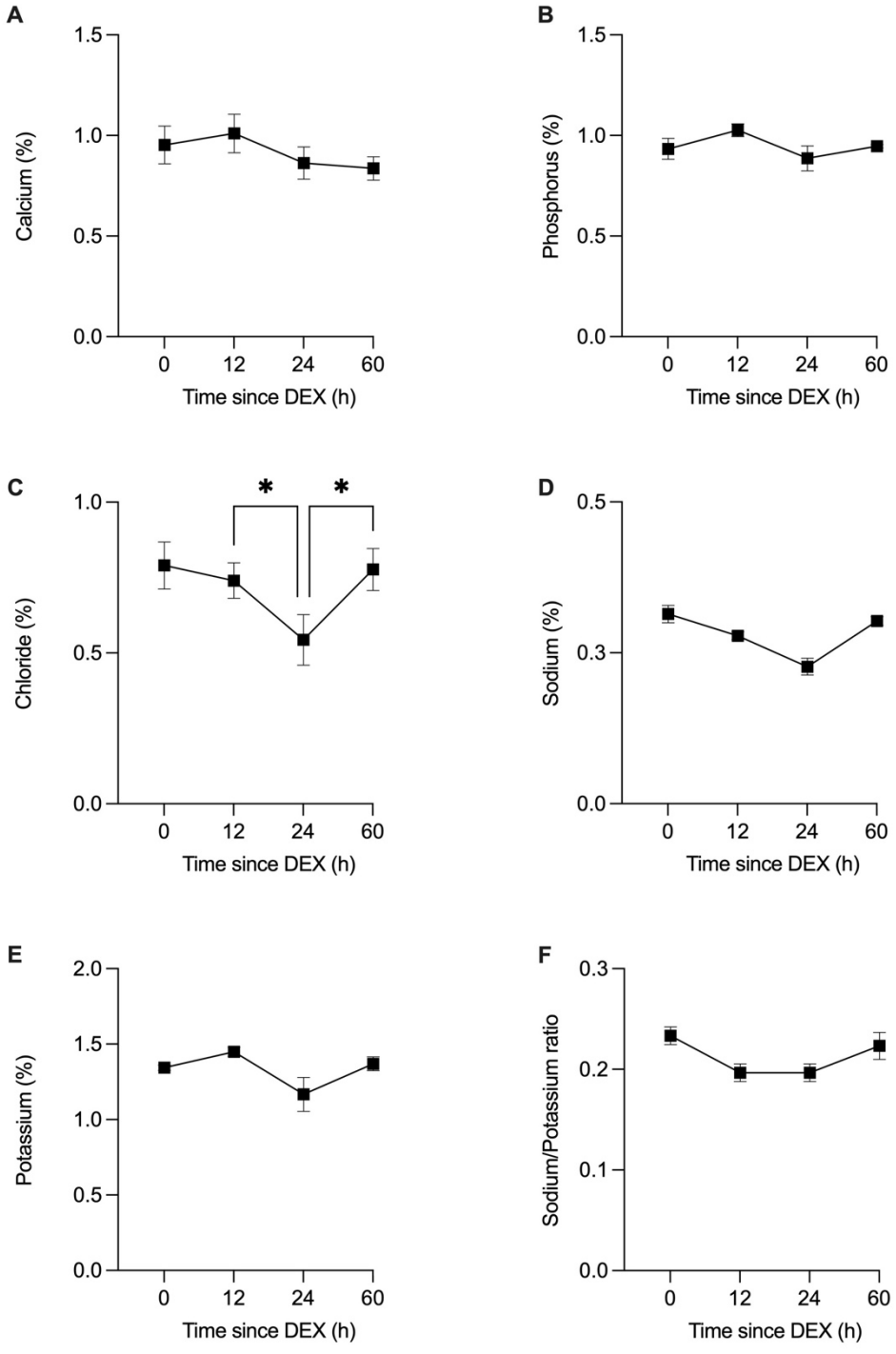

A
Calcium (%)
1.5
1.0
0.5
0.0
0
12
24
60
Time since DEX (h)
B
Phosphorus (%)
1.5
1.0
0.5
0.0
0
12
24
60
Time since DEX (h)
C
Chloride (%)
*
*
1.0
0.5
0.0
0
12
24
60
Time since DEX (h)
D
Sodium (%)
0.5
0.3
0.0
0
12
24
60
Time since DEX (h)
E
Potassium (%)
2.0
1.5
1.0
0.5
0.0
0
12
24
60
Time since DEX (h)
F
Sodium/Potassium ratio
0.3
0.2
0.1
0.0
0
12
24
60
Time since DEX (h)

FIGURE 4

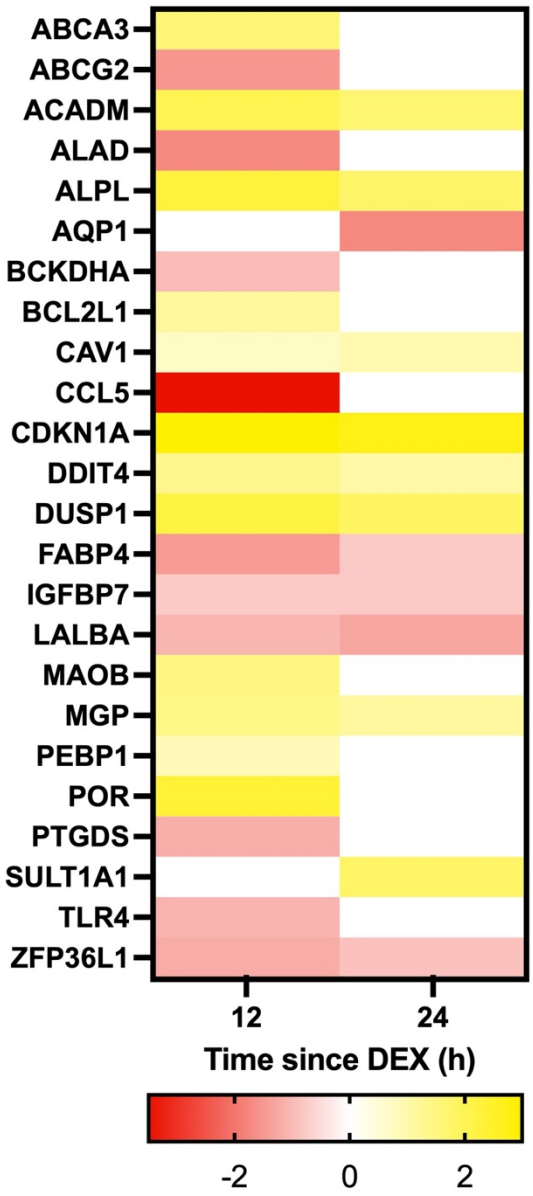

ABCA3
ABCG2
ACADM
ALAD
ALPL
AQP1
BCKDHA
BCL2L1
CAV1
CCL5
CDKN1A
DDIT4
DUSP1
FABP4
IGFBP7
LALBA
MAOB
MGP
PEBP1
POR
PTGDS
SULT1A1
TLR4
ZFP36L1
12
24
Time since DEX (h)
-2
0
2

FIGURE 5

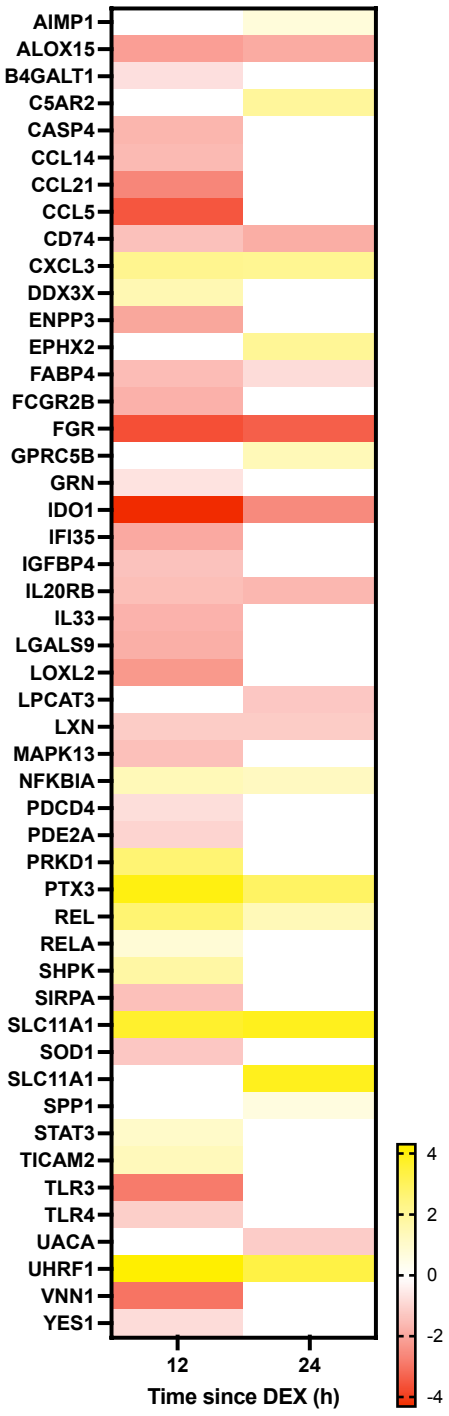
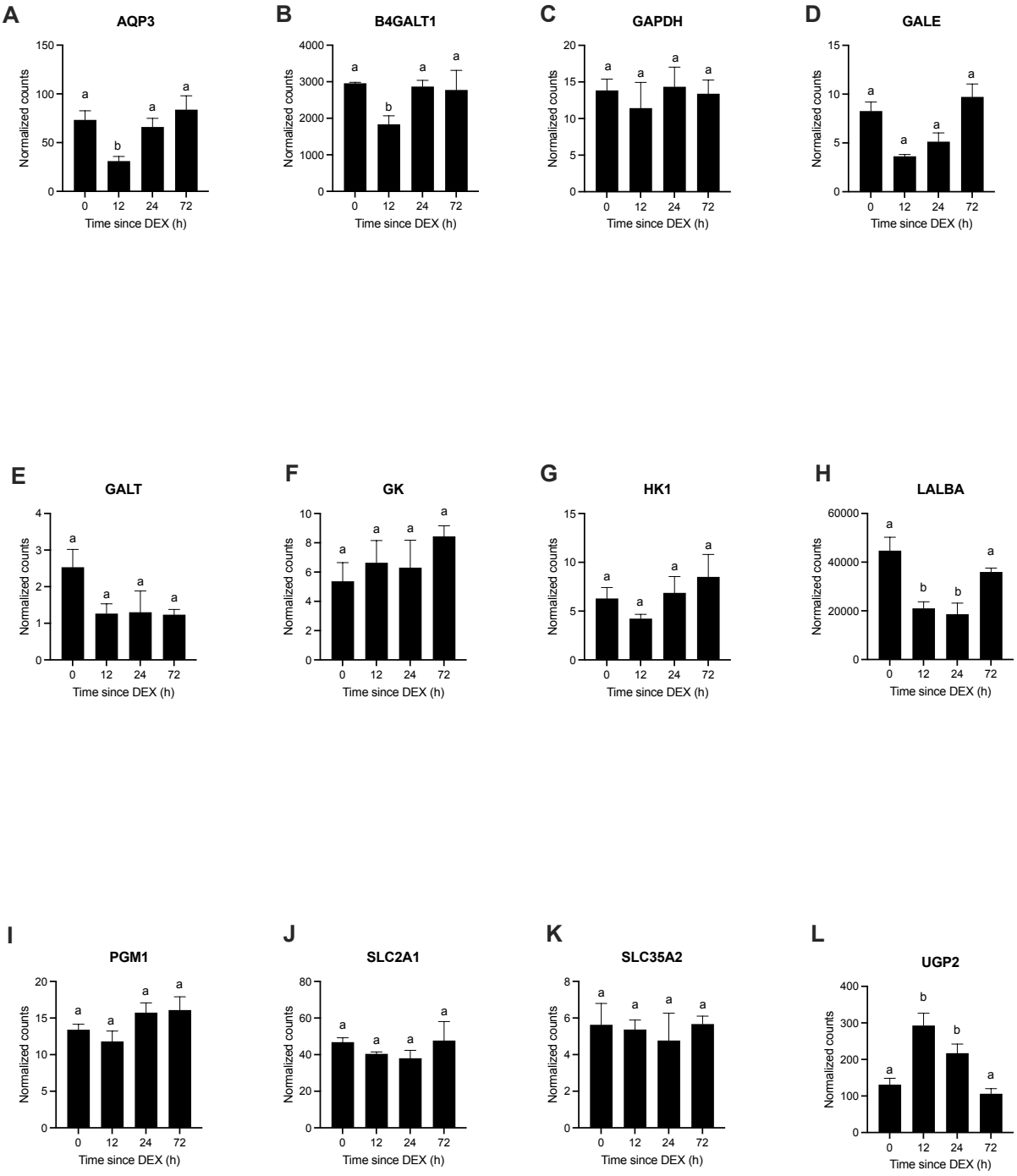
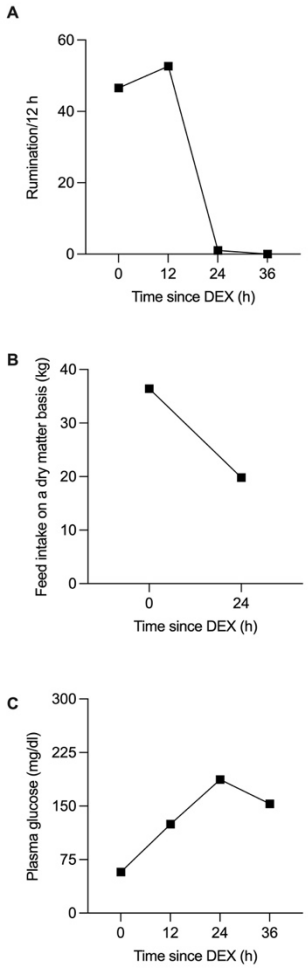

AIMP1
ALOX15
B4GALT1
C5AR2
CASP4
CCL14
CCL21
CCL5
CD74
CXCL3
DDX3X
ENPP3
EPHX2
FABP4
FCGR2B
FGR
GPRC5B
GRN
IDO1
IFI35
IGFBP4
IL20RB
IL33
LGALS9
LOXL2
LPCAT3
LXN
MAPK13
NFKBIA
PDCD4
PDE2A
PRKD1
PTX3
REL
RELA
SHPK
SIRPA
SLC11A1
SOD1
SLC11A1
SPP1
STAT3
TICAM2
TLR3
TLR4
UACA
UHRF1
VNN1
YES1
12
24
Time since DEX (h)
4
2
0
-2
-4

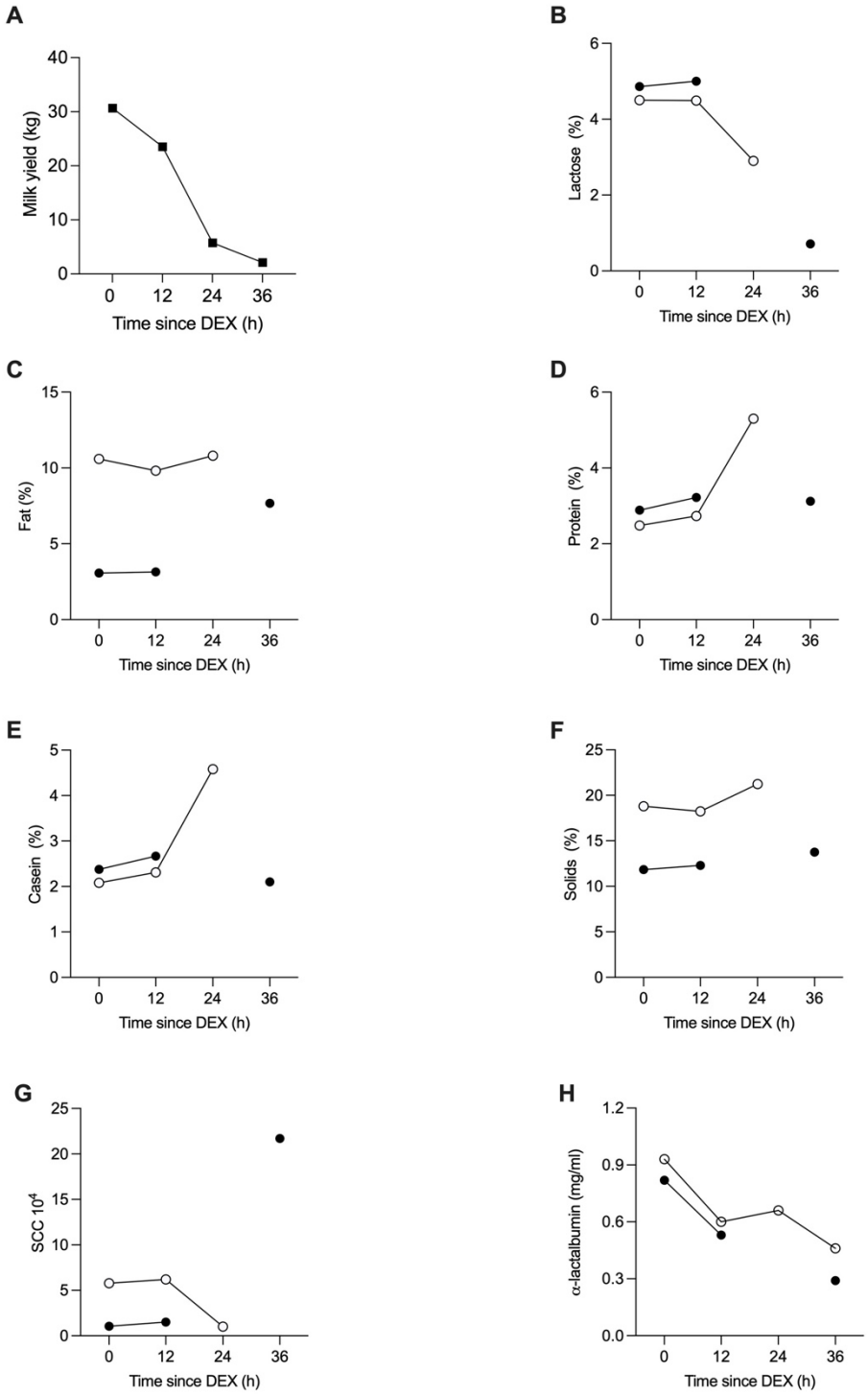
FIGURE 6


A
AQP3
B
B4GALT1
C
GAPDH
D
GALE
E
GALT
F
GK
G
HK1
H
LALBA
I
PGM1
J
SLC2A1
K
SLC35A2
L
UGP2
Normalized counts
Time since DEX (h)
0
12
24
72

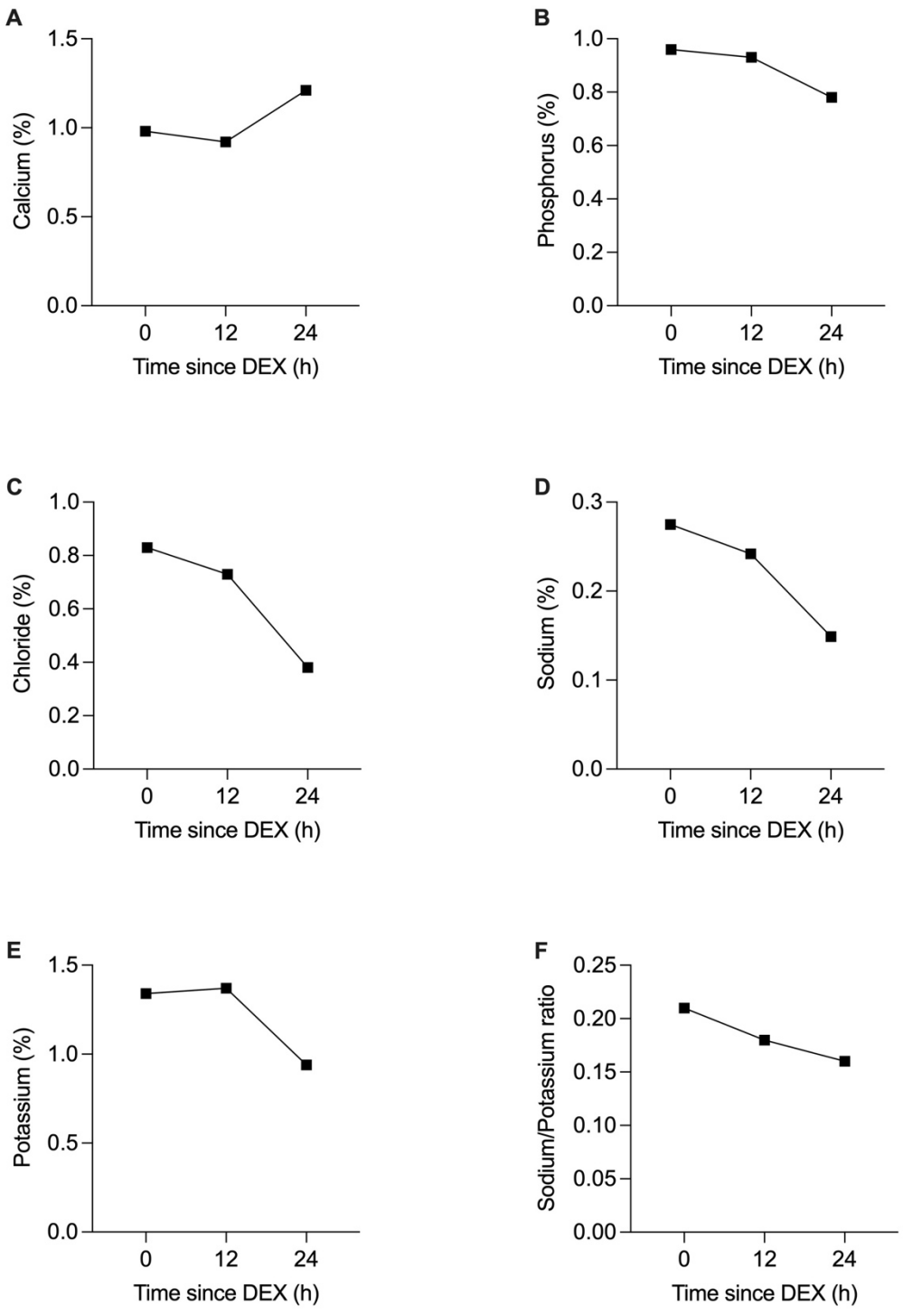
SUPPLEMENTARY FIGURE 1



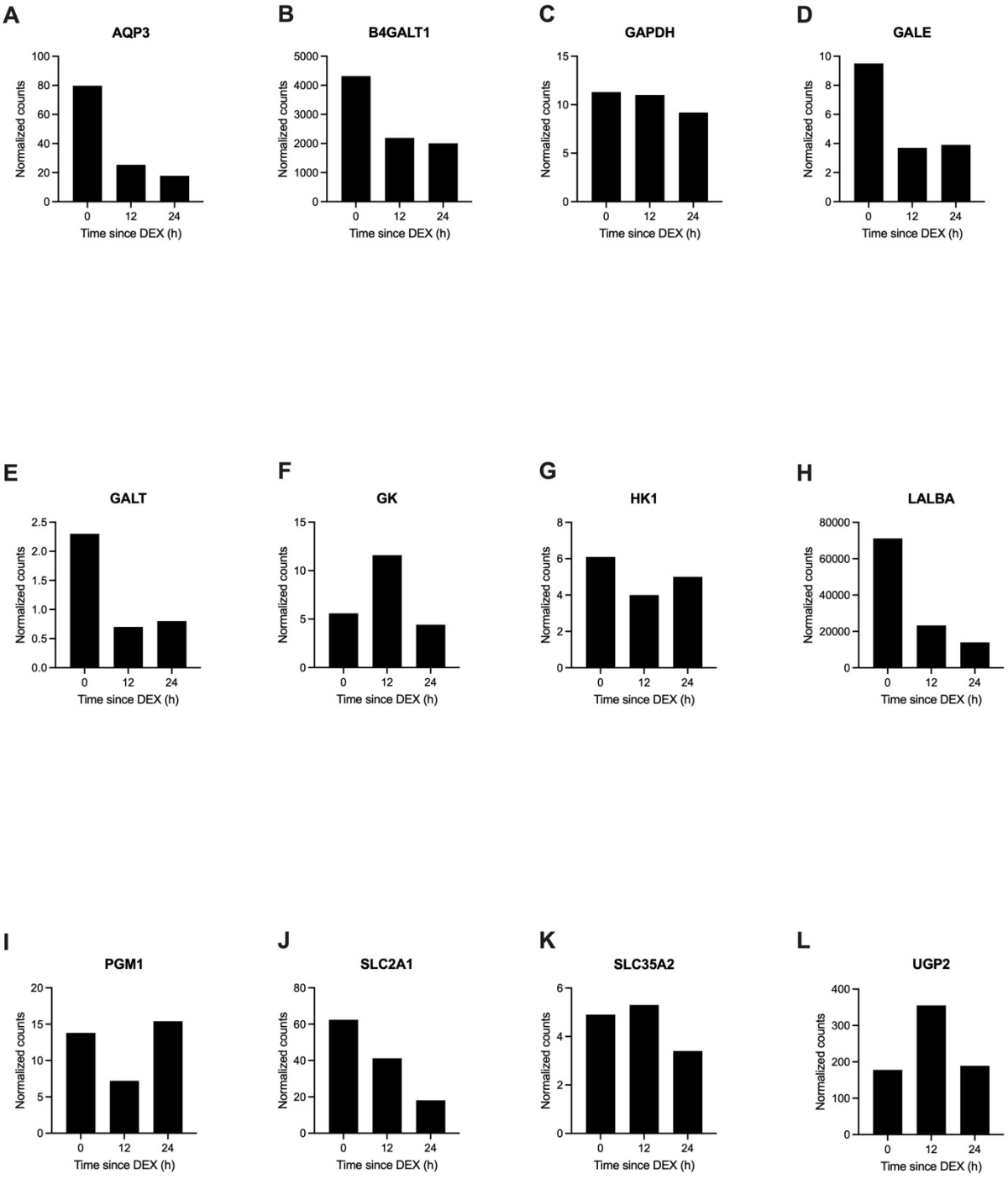
SUPPLEMENTARY FIGURE 2



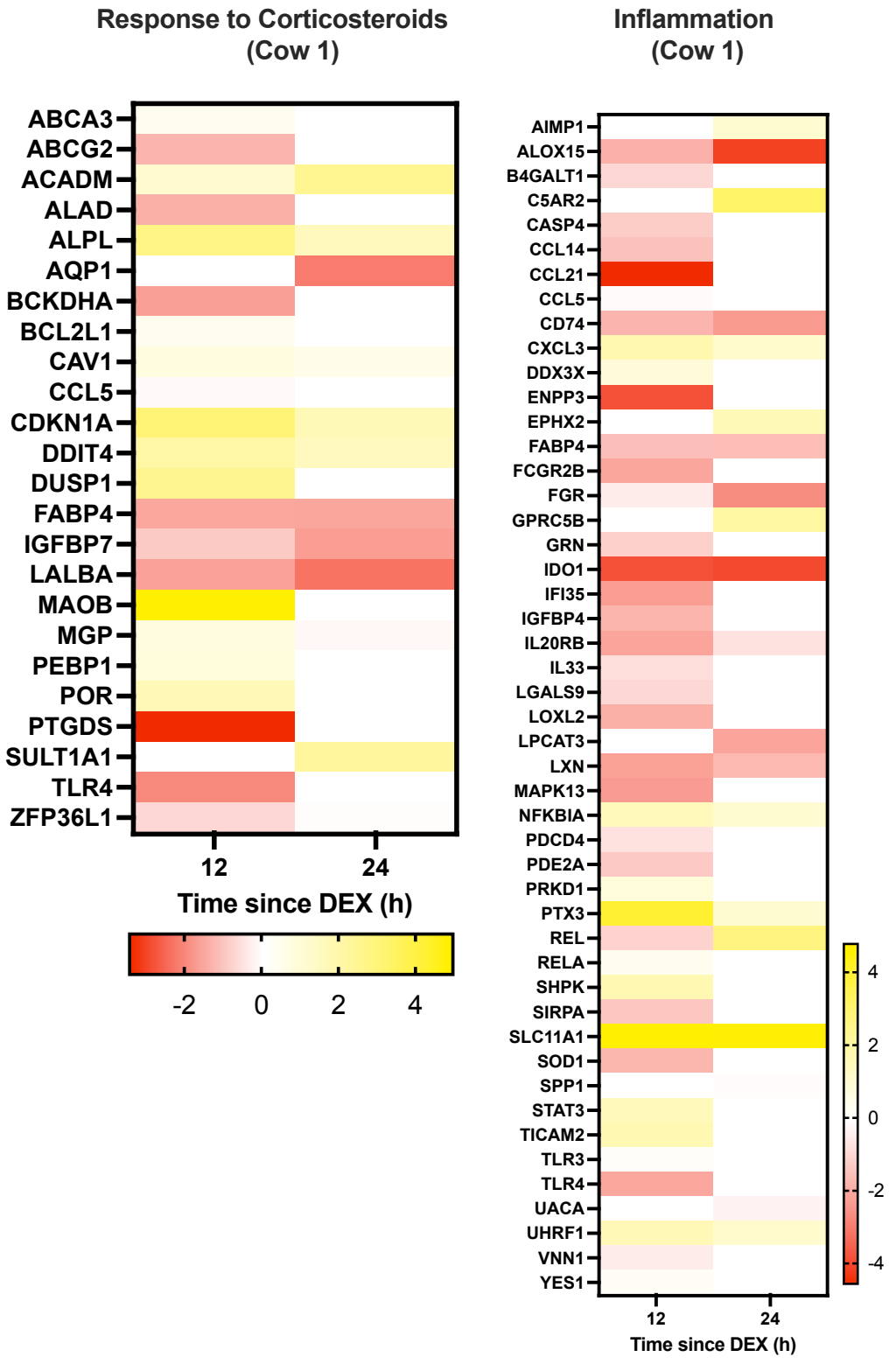
SUPPLEMENTARY FIGURE 3



SUPPLEMENTARY FIGURE 4



SUPPLEMENTARY FIGURE 5



Chapter 4. Translational potential of the murine mammary explant system for the study of glucocorticoid regulation of alpha-lactalbumin gene expression

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ABSTRACT

The biological mechanism by which maternal stress suppresses lactation has not been defined. While glucocorticoids (GC) are required for milk synthesis, excessive endogenous and exogenous GCs suppress milk production. We recently demonstrated that a single high dose of a synthetic GC administered to lactating dairy cows transiently suppressed milk volume, milk lactose content, milk α-lactalbumin (LALBA) content, and *Lalba* gene expression. The LALBA protein is required for lactose synthesis, but its hormonal regulation remains poorly understood. The primary objective of this work was to determine the reliability and replicability of the murine mammary explant system for the study of GC-mediated regulation of *Lalba* expression. We defined two doses of corticosterone (0.01 and 1.0 µg/ml, CORT) at which *Lalba* expression could be maximally upregulated (0.01 µg/ml), inhibited (1.0 µg/ml), or suppressed following initial stimulation. We also interrogated the changes in *Csn2, B4galt1, Ugp2, N3rc1, Stat5a, Fkbp5* expression in response to CORT. We demonstrated for the first time that *Lalba* expression in mammary explants from midpregnant mice can be suppressed by over 50% in response to a high dose of CORT after initial 48 h of stimulation by a low dose of CORT.

INTRODUCTION

The increase in milk production at the start of lactation requires the rapid upregulation of milk protein gene expression, including the transcription of α-lactalbumin (LALBA) [1]. The LALBA protein is required for the synthesis of lactose, where the transcriptional control and synthesis of LALBA are strongly correlated with overall milk volume [1]. As a case in point, pups born to *Lalba* knock out dams failed to thrive because the milk was viscous with low water content [2]. Conversely, naturally occurring mutations in the *Lalba* promoter are associated with increased milk LALBA protein content, milk lactose content, and overall milk production by Holstein cows [3,4].

The hormonal requirements for *Lalba* expression were studied *ex vivo* using mammary explants from mice and rats in the 1970s and 80s [5]. As it turns out, the modulation of *Lalba* expression by glucocorticoids (GC) is different from that of milk protein genes such as β-casein (Csn2), β-1,4-galactosyltransferase 1 (B4galt1), and whey acidic protein. For example, the dose-response relationship between GC concentration and *Csn2* expression is classically sigmoidal and monotonic. By contrast, a low concentration of GC stimulated *Lalba* expression while a high concentration of GC suppressed it [6,7]. This non-monotonic, biphasic regulation of *Lalba* expression by GC aligns with our findings and the work of others where exogenous GCs administered to lactating dairy cows suppressed milk production, milk lactose and LALBA protein content, and *LALBA* gene expression, without affecting milk fat and casein content [8,9].

The abundant synthesis and secretion of the LALBA protein and lactose cannot be faithfully recapitulated in mammary epithelial cell lines or primary cell cultures [5]. Mammary explants maintain their intricate alveolar architecture *ex vivo* and have been used to demonstrate the biphasic effect of GC on *Lalba* expression [5]. Yet, questions remain whether the mouse mammary explant system is a suitable *ex vivo* model for the study of GC regulation of milk, lactose, and LALBA synthesis. For this reason, the primary objective of this work was to determine

the reliability and replicability of the murine mammary explant system for the study of GC regulation of *Lalba* expression. The secondary objective was to determine the translational potential of the murine mammary explant system by comparing changes in the expression of specific genes in this *ex vivo* system to those observed in dairy cows [9].

MATERIALS AND METHODS

Animals

Female Balb/cJ mice (Jackson Laboratories) between 6 weeks and 3 months of age were used. Animals were housed in the UC Davis Cole B mouse facility in a room with a 14:10 light cycle. Mice had *ad libitum* access to commercial feed, water, and enrichment. Animal health, room humidity and temperature were monitored daily. The presence of a vaginal plug was recorded as day 0 of pregnancy. Necropsy was performed on day 11 or 12 of pregnancy for all experiments. Animals were euthanized using CO_2 and cervical dislocation. The UC Davis Animal Use Committee approved the protocol (Study # 22190).

Explant and Culture

Mammary gland tissue (~500 mg) from each mouse (variable glands, but always including the 4th abdominal) was removed at necropsy and pooled prior to mincing into explants (approximately 10 mg and 8 mm^3). Explants (30-50 mg per well) were floated at the gas:medium interface on siliconized lens paper in serum-free culture in a 50% O_2:95% CO_2 humidified incubator. Explants were cultured in Medium 199 (Gibco^{TM} Thermofisher Scientific) with L-glutamine, phenol red, Earle's salts, 2.2 mg/ml of sodium bicarbonate, penicillin, streptomycin, in the presence of bovine insulin (INS, 1 µg/ml, Sigma-Aldrich) and ovine prolactin (PRL, 1 µg/ml, Sigma-Aldrich) and varying concentrations of dexamethasone (DEX, Sigma-Aldrich) or corticosterone (CORT, Genesse Scientific Corp) for up to 72 hours (h). Media was changed every 24 h.

RNA isolation and purification

Fresh non-explanted tissue (approximately 100 mg) was flash frozen to determine baseline gene expression. Total RNA was isolated from fresh and explanted tissue using TRIzol (ThermoFisher Scientific) and 1-bromo-3-chloropropane. Total RNA (5 µg) was DNase-treated per the manufacturer's instructions (Sigma-Aldrich). RNA integrity and yield were confirmed by formaldehyde gel electrophoresis and spectrophotometry (Nanodrop, ThermoFisher Scientific) before and after DNase treatment. RNA (1µg) was reverse transcribed and the cDNA diluted 10-fold. Reverse transcription negative controls and a no-template control were subjected to real-time PCR (Applied Biosystems) using 18s rRNA primers and GoTaq™ Green MasterMix (ThermoFisher Scientific) to confirm a successful reverse transcription reaction and DNase treatment before proceeding to quantitative PCR.

Quantitative PCR

Primers for murine *Lalba*, *Csn2*, *B4galt1*, UDP-Glucose Pyrophosphorylase 2 (*Ugp2*), Signal Transducer and Activator of Transcription 5A (*Stat5a*), FKBP Prolyl Isomerase 5 (*Fkbp5*), Nuclear Receptor Subfamily 3 Group C Member 1 (*Nr3c1*), 18S ribosomal RNA (rRNA), Mitochondrial Ribosomal Protein L39 (Mrpl39), and Apoptosis Inhibitor 5 (Api5) genes were used (Table 1). Sequences for all primer products were verified at the UC Davis DNA sequencing core. Melt curve analysis showed a single amplification product for each primer pair (0.4 µL of 10 mM per primer pair per reaction) when cDNA (2 µL) was amplified using fast SYBR Green Master Mix (Applied Biosystems). The mRNA expression levels were normalized to those for 18s rRNA, Mrpl39, and Api5 using relative standard curves constructed from five- or four-fold serial dilutions of cDNA from involuting or explanted, respectively, murine mammary tissue.

Statistical analyses

Graphpad Prism (V 9.3.0, Graphpad Prism Software, LLC) was used for all statistical analyses. A two-way or one-way mixed effects analysis of variance or Kruskal-Wallis test, as

appropriate, with a *post-hoc* multiple comparisons test, was performed to determine differences in gene expression between treatments or timepoints. Significance was set at a *P* value of .05.

RESULTS

Identification of stimulatory and inhibitory DEX and CORT doses for Lalba expression

To identify stimulatory and inhibitory doses of DEX and CORT for *Lalba* expression, four experiments (A-D) were performed with varying doses of DEX (0, 0.0001, 0.001, 0.01, 0.1 μg/ml) and CORT (0, 0.001, 0.01, 0.1, 1 μg/ml). In Experiments A, B, and C, all explants were removed at 48 h. The time points and doses were chosen based on previous literature and preliminary validation work in our lab [6,10,11].

The effect of DEX on *Lalba* expression was not consistent across experiments A-C. In experiment A, the average expression of *Lalba* (Fig 1A) was 5.5-fold (*P* = .03) and 5.1-fold higher (*P* = .03) at the 0.0001 μg/ml dose compared to 0 and 0.1 μg/ml DEX, respectively, but there was no difference in *Lalba* expression at the 0 and 0.1 μg/ml DEX doses. In Experiment B (Fig 2A), there were no differences in *Lalba* expression between DEX treatments. In Experiment C (Fig 3A), *Lalba* expression in explants exposed to 0.0001 μg/ml DEX was no different than that in explants receiving 0 μg/ml DEX. Higher doses of DEX (0.001, 0.01, 0.1 μg/ml) suppressed (*P* < .05) *Lalba* expression compared to the 0 μg/ml DEX dose (Fig 3A).

The effect of CORT on *Lalba* expression in Experiments A-C was more consistent than that of DEX. The average expression of *Lalba* (Fig 1B) in Experiment A was 5.6-fold higher (*P* = .0003) at the 0.001 μg/ml dose compared to no CORT. Likewise, exposure of explants to a 0.001 μg/ml CORT dose resulted in *Lalba* mRNA levels that were 4.5- (*P* = .0004), 7.5- (*P* = .0002), and 9-fold (*P* = .0002) higher than at the 0.01, 0.1, and 1.0 μg/ml doses, respectively. There was no difference in *Lalba* expression between CORT treatments in Experiment B (Fig 2B). In Experiment

C, average *Lalba* mRNA levels (Fig 3B) in explants receiving the 0.01 μg/ml dose were 6.1-fold higher (P = .05) compared to 1.0 μg/ml CORT.

From experiments A-C, it was not possible to find a consistent dose for DEX at which *Lalba* expression was stimulated. For this reason, only CORT was used in Experiment D. In Experiment D, explants in two wells were first exposed to 0.001 μg/ml for 48 h and at the media change were switched to a 1.0 μg/ml CORT dose. Explants were removed at 24, 48 and 72 h. In Experiment D, *Lalba* expression was 10-fold higher (P = .017) in explants exposed to 0.01 μg/ml compared to those receiving 1.0 μg/ml CORT dose at 48 h. The expression of *Lalba* in explants that received the 1.0 μg/ml CORT dose after 48 h of exposure to the stimulatory dose was ~60% lower at 72 h compared to 24 h (P = .02). The *Lalba* mRNA levels in explants exposed to 0.001 μg/ml CORT were variable at 48 and 72 h and not significantly different from *Lalba* mRNA levels in explants exposed to 0 μg/ml CORT. Based on the results of Experiments A-D and the work of previous investigators, the 0.01 μg/ml CORT dose was selected for subsequent experiments [6,10,11].

Effect of a high dose of CORT on Lalba expression after initial exposure to a low CORT dose

Experiment E (Fig 5) tested a stimulatory CORT dose of 0.01 μg/ml and an inhibitory CORT dose of 1 μg/ml. Explants were removed at 24, 48, 60, and 72 h. Media was switched in nine wells at 48 h from the stimulatory to the inhibitory CORT dose. Among explants continuously exposed to the stimulatory dose, the *Lalba* expression increased 1.86-fold (P = .03) between 24 and 48 h (Fig 5A). The expression of *Lalba* at 60 h differed by treatment (P < .0001), with *Lalba* expression in explants exposed to 0.01 μg/ml CORT 7.2-fold (P < .0001) higher than that of explants exposed to 1.0 μg/ml CORT continuously. The expression of *Lalba* in explants exposed to only 12 h of the inhibitory dose at 60 h was 2.5-fold lower (P = .0012) than those continually receiving the stimulatory dose for 60 h. In fact, there was a 2-fold decrease (P = .02) in *Lalba* expression within 12 h of switching from 0.01 to 1.0 μg/ml CORT. The difference in *Lalba*

expression between samples continually exposed to the inhibitory dose for 60 h or only for 12 h was not significant. While relative *Lalba* mRNA quantity at the 0 and 0.01 μg/ml CORT doses was not significantly different at 60 h, *Lalba* expression in explants receiving 0 μg/ml CORT did not increase between 60 and 72 h. By contrast, in explants continually exposed to the 0.01 μg/ml CORT dose, *Lalba* expression increased 1.2-fold between 60 and 72 h such that by 72 h the difference between *Lalba* expression in explants continually exposed to 0.01 μg/ml and no CORT became significant (P = .02). Mean *Lalba* expression in samples continually exposed to the inhibitory dose remained low between 60 and 72 h and was 9-fold lower (P = .0002) than in those samples continuously exposed to the stimulatory dose at 72 h. The expression of *Lalba* in explants exposed to only 24 h of the inhibitory dose was 3.74-fold lower (P = .001) than the expression of *Lalba* exposed to the stimulatory dose continually.

In Experiment F, explants were exposed to 0.01, 1.0, or 0 μg/ml CORT and removed at 48 or 60 h. Three wells received a stimulatory CORT dose for 48 h followed by 12 h of an inhibitory CORT dose. The expression of *Lalba* (Fig 6A) in the three wells decreased by 56% (P = .025) following exposure to the inhibitory CORT dose. The expression of *Lalba* was 10.6-fold higher (P = .0002) in explants continually exposed to the stimulatory CORT dose compared to those exposed to 0 and 1.0 μg/ml CORT at 60 h. There was no difference in *Lalba* mRNA levels between explants continually exposed to the inhibitory CORT dose and those with only 12 h of exposure. The expression of *Lalba* in explants continually exposed to 0.01 μg/ml was 2.3-fold higher (P = .004) compared to *Lalba* mRNA levels in explants exposed to 12 h of the inhibitory dose. In summary, it appears that *Lalba* expression can be suppressed by more than 50% within 12 h of exposure to an inhibitory CORT dose of 1.0 μg/ml in mammary explants from midpregnant mice that were previously exposed to 48 h of a stimulatory 0.01 μg/ml CORT dose.

Effect of GC on Csn2, B4galt1, Ugp2, Nr3c1, Stat5a, and Fkbp5 expression

The expression of *Csn2* (Fig 1C and 1D; P < .05) and *B4galt1* (Fig 1E and 1F; P < .05) increased in a dose-dependent manner in response to DEX and CORT in Experiment A. There

was no effect of DEX or CORT on *Csn2* and *B4galt1* expression in Experiment B (Fig 2C and 2E). In Experiment C, there was a significant dose-dependent effect of DEX on *Csn2* (Fig 3C; $P = .0002$) and *B4galt1* (Fig 3E; $P < .0001$) expression. In Experiment D, the dose-dependent increase in *Csn2* expression in response to CORT approached significance (Fig 4C; $P = .06$), but there was no difference in *B4galt1* expression.

In Experiment E, overall *Csn2* expression (Fig 5B) increased over time ($P < .0001$) with differences in expression between treatments ($P = .009$). The expression of *Csn2* in response to the switch from 0.01 to 1.0 µg/ml CORT increased 1.9-fold ($P = .07$) between 24 and 60 h. The overall expression of *B4galt1* (Fig 5C) increased over time ($P = .0006$) with differences in expression between treatments ($P < .0001$). At 24 h, there was no difference in *B4galt1* expression between treatments, but at 48 h the expression of *B4galt1* in explants receiving the 1.0 and 0.01 µg/ml CORT dose was 3.1-fold ($P = .02$) and 2.3-fold ($P = .005$) higher, respectively, than that in explants receiving 0 µg/ml CORT. At 60 h, *B4galt1* mRNA levels increased 1.6-fold in explants that were switched to the 1.0 µg/ml dose and were not different from that in explants that had been receiving the 1.0 µg/ml dose continually. At 72 h, the *B4galt1* expression levels were higher in explants receiving 1.0 µg/ml CORT continually ($P < .01$) or for 24 h ($P < .01$) than those receiving no CORT.

In Experiment F, *Csn2* expression (Fig 6B) increased in response to time ($P = .07$) and increasing CORT dose ($P = .009$). There was no difference in *Csn2* mRNA levels between treatments at 48 h. At 60 h, there was no difference in *Csn2* expression between the 0.01 and 1.0 µg/ml CORT doses. After switching the media from 0.01 to 1.0 µg/ml CORT, *Csn2* expression increased 2-fold ($P = .03$). The expression of *B4galt1* (Fig 6C) increased in response to time ($P = .005$) and increasing CORT dose ($P = .01$). There was no difference in *B4galt1* mRNA levels between treatments at 48 h. At 60 h, there was no difference in *B4galt1* expression between the 0.01 and 1.0 µg/ml CORT doses. After switching the media from 0.01 to 1.0 µg/ml CORT, *B4galt1* expression increased 1.96-fold ($P = .007$). The expression of *B4galt1* was 2.2-fold higher ($P =$

.0009) in those explants that were exposed to only 12 h of 1.0 μg/ml CORT compared to those that received no CORT.

Next, we wanted to determine whether the expression of candidate genes selected from our *in vivo* experiment where lactating dairy cows received a single, high dose of DEX would follow the same expression pattern in explants as was observed the udder. We selected a rate-limiting gene in the lactose synthesis pathway, *Ugp2*, a transcription factor (*Stat5a*) which binds activated GC receptor (GR) to stimulate *Lalba* expression, a GC-responsive gene *(Fkbp5),* and the gene encoding the GR (*N3rc1*) [1,12]. The expression of *Ugp2*, *Nr3c1*, *Stat5a*, and *Fkbp5* was measured in all samples from Experiment E. There was a significant effect of time ($P = .0003$) and treatment ($P < .0001$) on *Fkbp5* expression (Fig 7C). In explants that were switched to the 1.0 μg/ml CORT dose, the *Fkbp5* mRNA levels increased 6.6-fold within 12 h ($P = .02$), reaching the same levels as that in explants continually receiving 1.0 μg/ml CORT. There was no difference in the expression of *Ugp2* (Fig 7A)*, Nr3c1* (Fig 7B), or *Stat5a* (Fig 7D) over time or between treatments, except that the expression of *Stat5a* decreased by ~30% ($P = .004$) between 24 h and 72 h following the transition from 0.01 to 1.0 μg/ml CORT.

DISCUSSION

In this study, we defined two doses (0.01 and 1.0 μg/ml) of CORT at which *Lalba* expression could be maximally upregulated, inhibited, or suppressed following initial stimulation. We also interrogated the changes in gene expression for the milk protein gene, *Csn2*, two rate-limiting genes in the lactose synthesis pathway, *B4galt1* and *Ugp2*, a transcription factor (*Stat5a*) which binds activated GC receptor (GR) to stimulate milk protein gene expression, and two GC-responsive genes (*Fkbp5* and *N3rc1*). We demonstrated for the first time that *Lalba* expression in mammary explants from midpregnant mice can be suppressed by over 50% in response to 1.0 μg/ml CORT after an initial 48 h of stimulation by 0.01 μg/ml CORT. The downregulation of *Lalba*

expression by 50% in response to the high GC dose is akin to our findings in dairy cows. Future research endeavors could focus on defining the mechanism by which *Lalba* expression is suppressed by GC using the murine mammary explant system, with a special focus on the regulatory landscape at the *Lalba* promoter in the first 12 h following the introduction of an inhibitory CORT dose to the media.

The effect of GC on *Csn2, Ugp2,* and *B4galt1* expression was not consistent between our murine mammary explant and *in vivo* dairy cow experiment. Akin to the findings of previous investigators who used murine mammary explants, there was a positive, dose-dependent effect of CORT on *Csn2* and *B4galt1* expression [7,13]. Yet, while *Csn2* and *B4galt1* expression increased in the explants in response to a change from low to high CORT, in our dairy cow experiment the mRNA levels of CSN2 did not change, but *B4galt1* expression decreased. Likewise, there was a discrepancy in the effect of a high GC dose on *Ugp2*, where its expression was upregulated in response to DEX in our dairy cow experiment, but there was no change in the explants in response to 1.0 µg/ml CORT. Factors that could have contributed to the discrepancy between the *in vivo* and *ex vivo* findings may be related to changes in blood flow in the udder and homeorhetic mechanisms that regulate plasma glucose levels [1,5]. Another explanation may be that the use of DEX in the dairy cow experiment affected only those intracellular pathways specific to GR, while the use of CORT resulted in activation of both the mineralocorticoid receptor (MR) and GR [14,15].

A GC can bind to either the MR or the GR, where the MR is responsible for orchestrating the effects of GC during basal metabolic activity, while in times of stress, GR activity is induced [16]. High levels of GC can downregulate *Nr3c1* mRNA expression, promote GR degradation, and impair GR translocation to the nucleus [17], yet in our dairy cow experiment, *Nr3c1* expression only tended to decrease by 12 h post-DEX (log-fold change -1.7, adj. P-value = .055) and there was no change in *N3rc1* mRNA levels in explants in response to increasing CORT dose. The lack of change in *Nr3c1* mRNA levels could also point to the important role of MR in the lactating

mammary gland, as it is thought that the MR compensates for GR loss or dysfunction during lactation [18–20].

Next, we investigated the change in *Fkbp5* gene expression, given that the FKBP5 protein is a key regulator of GR inactivation as part of an immediate negative feedback loop [12]. The mRNA levels of *Fkbp5* increased in both the murine mammary explants (6.6-fold, *P*=.02) and in the udder of the dairy cow (log-fold change 4.1, adjusted P-value 3.36×10^{-9}) within 12 h of high GC exposure. Recently, it was suggested that baseline cytosolic FKBP5 levels in the hippocampus are determined by MR, but in times of stress (i.e., high GC), GR activation drives further FKBP5 induction, thereby triggering a negative feedback loop and preventing activated GR translocation to the nucleus [21]. Combined, our findings regarding *Nr3c1* and *Fkbp5* expression in murine explants and the cow udder suggest that there is a need to define FKBP5, activated GR, and activated MR levels in response to GC in the mammary explants and then to correlate these findings with GR and MR binding to the *Lalba* promoter.

The *Lalba* promoter does not have consensus GR elements (GRE), but several putative GRE half-sites have been described in the 5' upstream region (945-950 bp), in intron 1 (1546-1511, 1590-1596, 1601-1606 bp), twice in intron 2 (1034-1939, 2161-2166 bp), and once after the termination site for RNA transcription (3763-3768 bp) in the rat *Lalba* gene [22–24]. It has been proposed that these GRE half-sites represent turn-on and turn-off sites by which GC-mediated biphasic regulation of *Lalba* is orchestrated [24]. Since the GR and MR share significant homology in the DNA and ligand binding domains and vary in the N-terminal binding domain [18,25,26], it is conceivable that activated MR binds to a specific GRE half-site to maintain *Lalba* transcription, while in times of stress, activated GR binds to a different GRE half-site to initiate suppression of *Lalba* expression [24]. The role of MR and GR in the biphasic regulation of gene expression has been demonstrated in rat primary neuronal cortical cells where at low levels, CORT was bound to the MR and stimulated brain derived neurotrophic factor (BDNF) gene expression whereas at high doses, CORT bound to GR and suppressed BDNF transcription [27].

Given that there are no known consensus GRE in the *Lalba* promoter, interrogating changes in transcription factors that are known to tether or heterodimerize with activated GR and MR may be necessary to define the regulatory landscape of *Lalba* transcription.

The putative GRE half-sites in the *Lalba* gene are proximal to CCAAT-enhancer-binding protein (C/EBP), nuclear factor 1 (NF1), and phosphorylated STAT5A binding sites [22,28]. Notably, there are also motifs (LA-1, 2, 3) unique to *Lalba* within the first 200 bp of the transcription start site that are conserved across species [5,22]. We sought to investigate changes in *Stat5a* mRNA levels given that this transcription factor forms a heterodimer with activated GR to promote whey acidic protein and *Csn2* gene expression and phosphorylated STAT5A has been shown to bind the murine *Lalba* promoter [28,29]. There were no changes in *Stat5a* gene expression in our *in vivo* dairy cow or the *ex vivo* mammary explant experiment. The lack of change in *Stat5a* gene expression could be explained by the fact that the GR-mediated regulation of *Lalba* expression might occur rapidly through changes in protein phosphorylation, transcription factor complex formation or dissociation, DNA binding, and epigenetic modification; therefore, a change in *Stat5a* gene expression would not be necessary to reduce *Lalba* mRNA levels. As such, the study of the regulatory landscape of *Lalba* transcription will need to be carefully considered as activated GR or MR may tether with transcription factors or modify access of transcription factors through histone modification [30].

CONCLUSION

In this study, we used a mammary explant system from midpregnant mice to define two doses of CORT at which *Lalba* expression could be stimulated, inhibited, or suppressed after an initial period of stimulation. Our findings regarding changes in *Lalba, Csn2,* and *B4galt1* expression in response to CORT aligned with those of previous investigators, where GC stimulated *Csn2* and *B4galt1* expression in a dose-dependent manner, but regulated *Lalba*

expression in a dose-biphasic way. Changing the media to 1.0 µg/ml CORT after 48 h of exposure of mammary explants to a 0.01 µg/ml CORT dose resulted in an increase in *Fkbp5* expression, without an effect on *Nr3c1* and *Stat5a* mRNA levels. The effect of CORT on *Lalba, Fkbp5*, *Nr3c1*, and *Stat5a* expression in the murine explant system was akin to the effect of DEX on the expression of those genes in the udder of lactating dairy cows. By contrast, there was a discrepancy in the effect of GC on *Csn2*, *Ugp2* and *B4galt1* expression between the murine mammary explants and the dairy cow udder. Future research endeavors should focus on identifying changes in activated GR and MR binding to GRE half-sites in the *Lalba* gene in murine mammary explants in response to stimulatory and inhibitory doses of GC.

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TABLES

TABLE 1

Table 1. Sequences of forward (F) and reverse (R) primers used for qPCR

Gene name	Sequence	T_m
Lalba F (5'-3')	CCCGAGTCGGAGAACATCTG	62
Lalba R (3'-5')	GGGCTTGTAGGCTTTCCAGT	62
Csn2 F (5'-3')	GGACTTGACAGCCCATGAAGG	60
Csn2 R (3'-5')	TAGCCTGGAGCACATCCTCT	60
B4galt1 F (5'-3')	GATGGACGACCGTAATGCCT	60
B4galt1 R (3'-5')	TGAGAGCAGAGACACCTCCA	60
Stat5a F (5'-3')	GGATACGTGAAGCCACAGATCAA	64
Stat5a R (3'-5')	CATGCTCTCATCCAGGTCAAACT	64
Ugp2 F (5'-3')	AGTCACAAACAAAACACGAGCA	60
Ugp2 R (3'-5')	GGCACTTGAGCGATTTCCAC	60
Fkbp5 F (5'-3')	AAACGGAAAGGCGAGGGATA	60
Fkbp5 R (3'-5')	ACACCACATCTCGGCAATCA	60
Nr3c1 F (5'-3')	CAACCTGACTTCCTTGGGGG	61
Nr3c1 R (3'-5')	TGGACGGAGGAGAACTCACA	61
Api5 F (5'-3')	AGTTGGGGCGAAAACTTCCA	61
Api5 R (3'-5')	TTTACCCTGGAGAGCCAAGC	61
Mrpl39 F (5'-3')	GGCCTGTCTTTACCCACGCACTT	61
Mrpl39 R (3'-5')	GGGTGGATTCGGTGTTCTCTGTCT	61
18s rRNA F (5'-3')	ACGGCTACCACATCCAAGGA	60
18s rRNA R (3'-5')	CCAATTACAGGGCCTCGAAA	60

FIGURE LEGENDS

Figure 1. In experiment A, duplicate wells of mammary explants from midpregnant mice were treated with 5 different doses of dexamethasone (DEX, left) and corticosterone (CORT, right). Samples were removed at 48 h. The expression of *Lalba* (A, B), *Csn2* (C, D), and *B4Galt1* (E, F) was normalized to three housekeeping genes (*Mrpl39, Api5, 18s rRNA*). Data are presented as the mean ± standard error of the mean. Different letters indicate significant differences ($P < .05$ in 1A, 1E, 1D, 1F; $P < .001$ in 1B) in mean relative mRNA levels between treatments.

Figure 2. In experiment B, duplicate wells of mammary explants from midpregnant mice were treated with 3 different doses of dexamethasone (DEX, left) and corticosterone (CORT, right). Explants were removed at 48 h. The expression of *Lalba* (A, B), *Csn2* (C, D), and *B4Galt1* (E, F) was normalized to three housekeeping genes (*Mrpl39, Api5, 18s rRNA*). Data are presented as the mean ± standard error of the mean. There were no significant differences between treatments.

Figure 3. In experiment C, triplicate wells of mammary explants from midpregnant mice were treated with 5 different doses of dexamethasone (DEX, left) and corticosterone (CORT, right). Explants were removed at 48 h. The expression of *Lalba* (A, B), *Csn2* (C, D), and *B4Galt1* (E, F) was normalized to three housekeeping genes (*Mrpl39, Api5, 18s rRNA*). Data are presented as the mean ± standard error of the mean. In 3A, different letters indicate significant differences ($P < .01$) in mean relative *Lalba* mRNA levels in comparison to a DEX dose of 0 ($P < .01$) and 0.0001 µg/ml ($P < .05$). In 3B, different letters indicate significant differences in mean relative *Lalba* mRNA levels between treatments in comparison to a CORT dose of 1.0 µg/ml ($P = .05$). In 3C, different letters indicate significant differences in mean relative *Csn2* mRNA levels in comparison to a DEX dose of 0 µg/ml (b, $P < .05$; c, $P < .0001$). In 3E, different letters indicate significant differences in

mean relative *B4galt1* mRNA levels in comparison to a DEX dose of 0 (P<.0001) and 0.0001 μg/ml ($P < .05$).

Figure 4. In experiment D, duplicate wells of mammary explants from midpregnant mice were treated with 5 different doses of corticosterone (CORT). Two wells that were treated with a 0.001 ug/ml CORT dose were switched to a 1.0 ug/ml CORT dose at 48 h. Explants were removed at 24, 48, and 72 h. The expression of *Lalba* (A), *Csn2* (B), and *B4Galt1* (C) was normalized to three housekeeping genes (*Mrpl39, Api5, 18s rRNA*). Data are presented as the mean ± standard error of the mean. In 4A, letters a, b, c indicate significant differences ($P < .05$) in mean relative *Lalba* mRNA levels between treatments at 48 h, the letters d, e, f indicate significant differences ($P < .05$) in mean relative *Lalba* mRNA levels between treatments at 72 h, and the * signifies a significant difference ($P < .05$) in *Lalba* mRNA levels following the switch from a 0.001 to 1.0 ug/ml CORT dose. In 4B, letters a, b, c, d indicate significant differences ($P < .01$) in mean relative *Csn2* mRNA levels between treatments at 48 h, the letters e, f, g, h indicate significant differences ($P < .01$) in mean relative *Csn2* mRNA levels between treatments at 72 h, and the * signifies a significant difference ($P < .05$) in *Csn2* mRNA levels following the switch from a 0.001 to 1.0 ug/ml CORT dose. In 4C, letters a and b indicate significant differences ($P < .05$) in mean relative *B4galt1* mRNA levels between treatments at 48 h, the letters c and d indicate significant differences (P<.01) in mean relative *B4galt1* mRNA levels between treatments at 72 h, and the ** signifies a significant difference ($P < .01$) in *B4galt1* mRNA levels following the switch from a 0.001 to 1.0 ug/ml CORT dose.

Figure 5. In experiment E, triplicate or quadruplicate wells of mammary explants from midpregnant mice were treated with 3 different doses of corticosterone (CORT). At 48 h, 9 wells that were treated with a 0.01 ug/ml CORT dose were switched to a 1.0 ug/ml CORT dose. Explants from 5 wells where the media was switched were removed at 60 h and the rest (n=4

wells) were removed at 72 h. All other explants treated in triplicate or quadruplicate at 0, 0.01, or 1.0 ug/ml CORT were removed at 24, 48, and 72 h. The expression of *Lalba* (A), *Csn2* (B), and *B4Galt1* (C) was normalized to three housekeeping genes (*Mrpl39, Api5, 18s rRNA*). Data are presented as the mean ± standard error of the mean. In 5A, letters a and b indicate significant differences ($P < .05$) in mean relative *Lalba* mRNA levels between time points for explants that received 48 h of 0.01 ug/ml CORT followed by 24 h of 1.0 ug/ml CORT. There were no significant differences in *Csn2* after the media switch from 0.01 to 1.0 ug/ml CORT (5B). In 5C, letters a and b indicate significant differences ($P < .01$) in mean relative *B4galt1* mRNA levels between time points for explants that received 48 h of 0.01 ug/ml CORT followed by 24 h of 1.0 ug/ml CORT. Significant differences in *Lalba, Csn2, and B4galt 1* expression between time points for explants receiving 0, 1.0, or 0.01 ug/ml CORT continuously are not shown in the figure. Refer to the manuscript body for additional information about differences in *Lalba*, *Csn2*, and *B4galt1* mRNA levels between treatments or time points.

Figure 6. In experiment F, duplicate or triplicate wells of mammary explants from midpregnant mice were treated with 3 different doses of corticosterone (CORT). At 48 h, 3 wells that were treated with a 0.01 ug/ml CORT dose were switched to a 1.0 ug/ml CORT dose. Explants were removed at 48 and 60 h. The expression of *Lalba* (A), *Csn2* (B), and *B4Galt1* (C) was normalized to three housekeeping genes (*Mrpl39, Api5, 18s rRNA*). Data are presented as the mean ± standard error of the mean. In 6A, letters a and b indicate significant differences ($P < .05$) in mean relative *Lalba* mRNA levels between treatments at 48 h, the letters c and d indicate significant differences ($P < .01$) in mean relative *Lalba* mRNA levels between treatments at 60 h, and the ** signifies a significant difference ($P < .01$) in *Lalba* mRNA levels following the switch from a 0.01 to 1.0 ug/ml CORT dose. In 6B, there were no differences between treatments at 48 h. Letters a, b, c indicate significant differences ($P < .05$) in mean relative *Csn2* mRNA levels between treatments at 60 h and the * signifies a significant difference ($P < .05$) in *Csn2* mRNA levels

following the switch from a 0.01 to 1.0 ug/ml CORT dose. In 6C, there were no differences in B4galt1 mRNA levels between treatments at 48 h. Letters a and b indicate significant differences ($P < .05$) in mean relative *B4galt1* mRNA levels between treatments at 60 h and the ** signifies a significant difference ($P < .01$) in *B4galt1* mRNA levels following the switch from a 0.01 to 1.0 ug/ml CORT dose.

Figure 7. In experiment E, triplicate or quadruplicate wells of mammary explants from midpregnant mice were treated with 3 different doses of corticosterone (CORT). At 48 h, 9 wells that were treated with a 0.01 ug/ml CORT dose were switched to a 1.0 ug/ml CORT dose. Explants from 5 wells where the media was switched were removed at 60 h and the rest (n=4 wells) were removed at 72 h. All other explants treated in triplicate or quadruplicate at 0, 0.01, or 1.0 ug/ml CORT were removed at 24, 48, and 72 h. The expression of *Ugp2* (A), *Nr3c1* (B), *Fkbp5* (C), and *Stat5a* (D) was normalized to three housekeeping genes (*Mrpl39, Api5, 18s rRNA*). Data are presented as the mean $\pm$ standard error of the mean. There were no significant differences between timepoints or treatments for Ugp2 (7A) or Nr3c1 (7B). In 7C, letters a and b indicate significant differences ($P < .05$) in mean relative *Fkbp5* mRNA levels between time points for explants that received 48 h of 0.01 ug/ml CORT followed by 24 h of 1.0 ug/ml CORT. In 7D, Letters a and b indicate significant differences ($P < .01$) in mean relative *Stat5a* mRNA levels between time points for explants that received 48 h of 0.01 ug/ml CORT followed by 24 h of 1.0 ug/ml CORT. Significant differences in gene expression between time points for explants receiving 0, 1.0, or 0.01 ug/ml CORT continuously are not shown in the figure. Refer to the manuscript body for information about differences in *Fkbp5* mRNA levels between treatments at each timepoint.

FIGURES

FIGURE 1

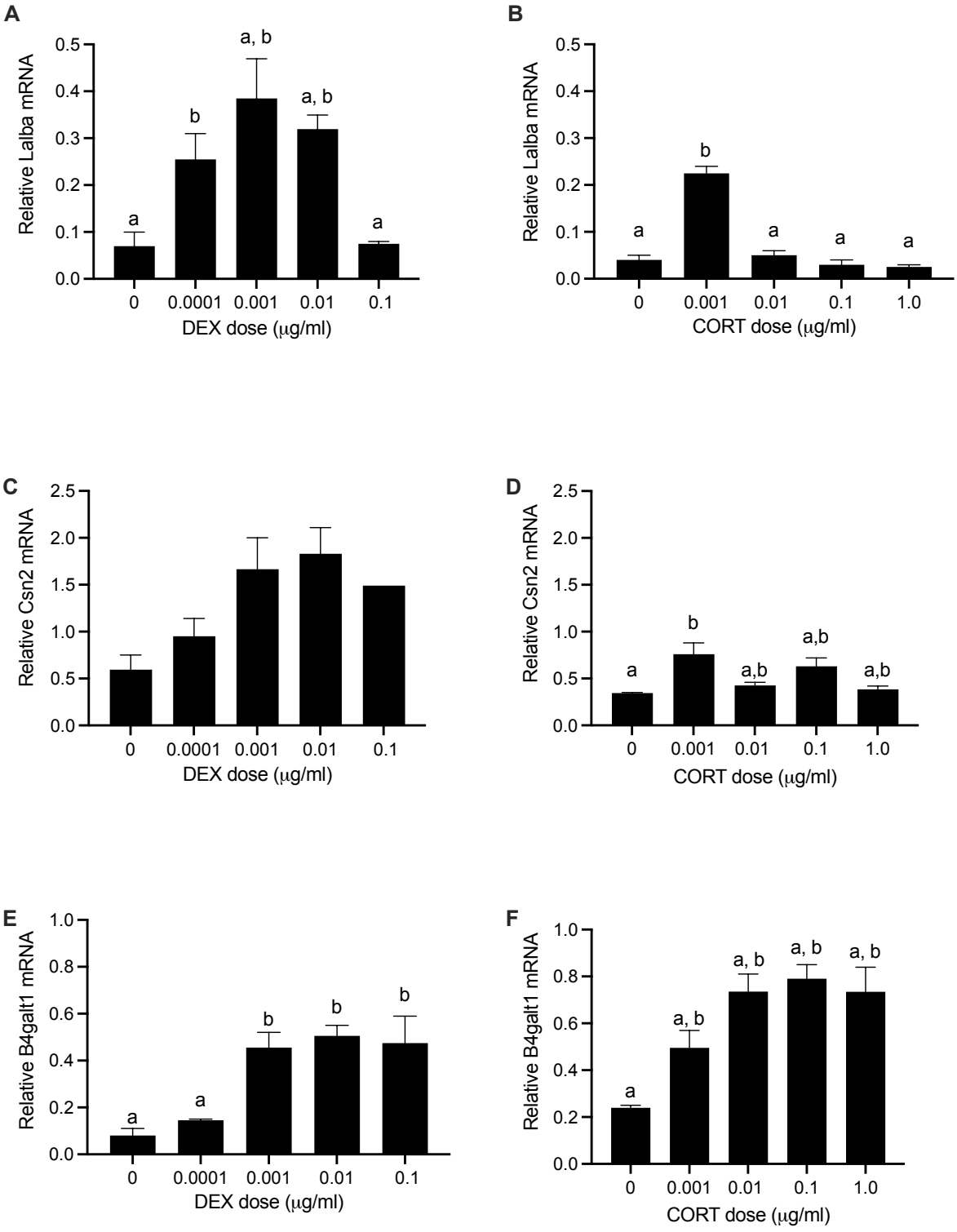


FIGURE 2

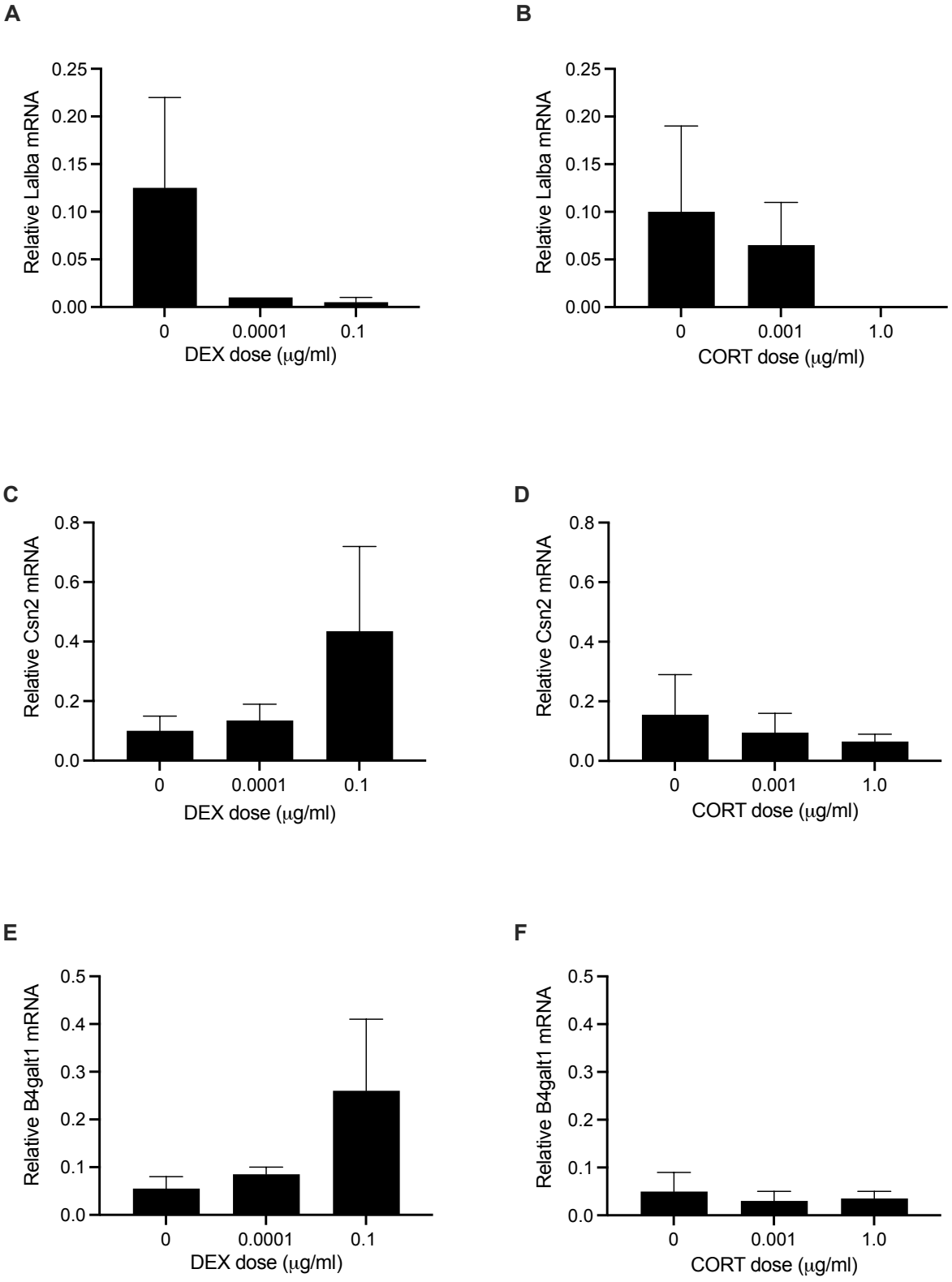

A
Relative Lalba mRNA
0.25
0.20
0.15
0.10
0.05
0.00
0
0.0001
0.1
DEX dose (μg/ml)
B
Relative Lalba mRNA
0.25
0.20
0.15
0.10
0.05
0.00
0
0.001
1.0
CORT dose (μg/ml)
C
Relative Csn2 mRNA
0.8
0.6
0.4
0.2
0.0
0
0.0001
0.1
DEX dose (μg/ml)
D
Relative Csn2 mRNA
0.8
0.6
0.4
0.2
0.0
0
0.001
1.0
CORT dose (μg/ml)
E
Relative B4galt1 mRNA
0.5
0.4
0.3
0.2
0.1
0.0
0
0.0001
0.1
DEX dose (μg/ml)
F
Relative B4galt1 mRNA
0.5
0.4
0.3
0.2
0.1
0.0
0
0.001
1.0
CORT dose (μg/ml)

FIGURE 3

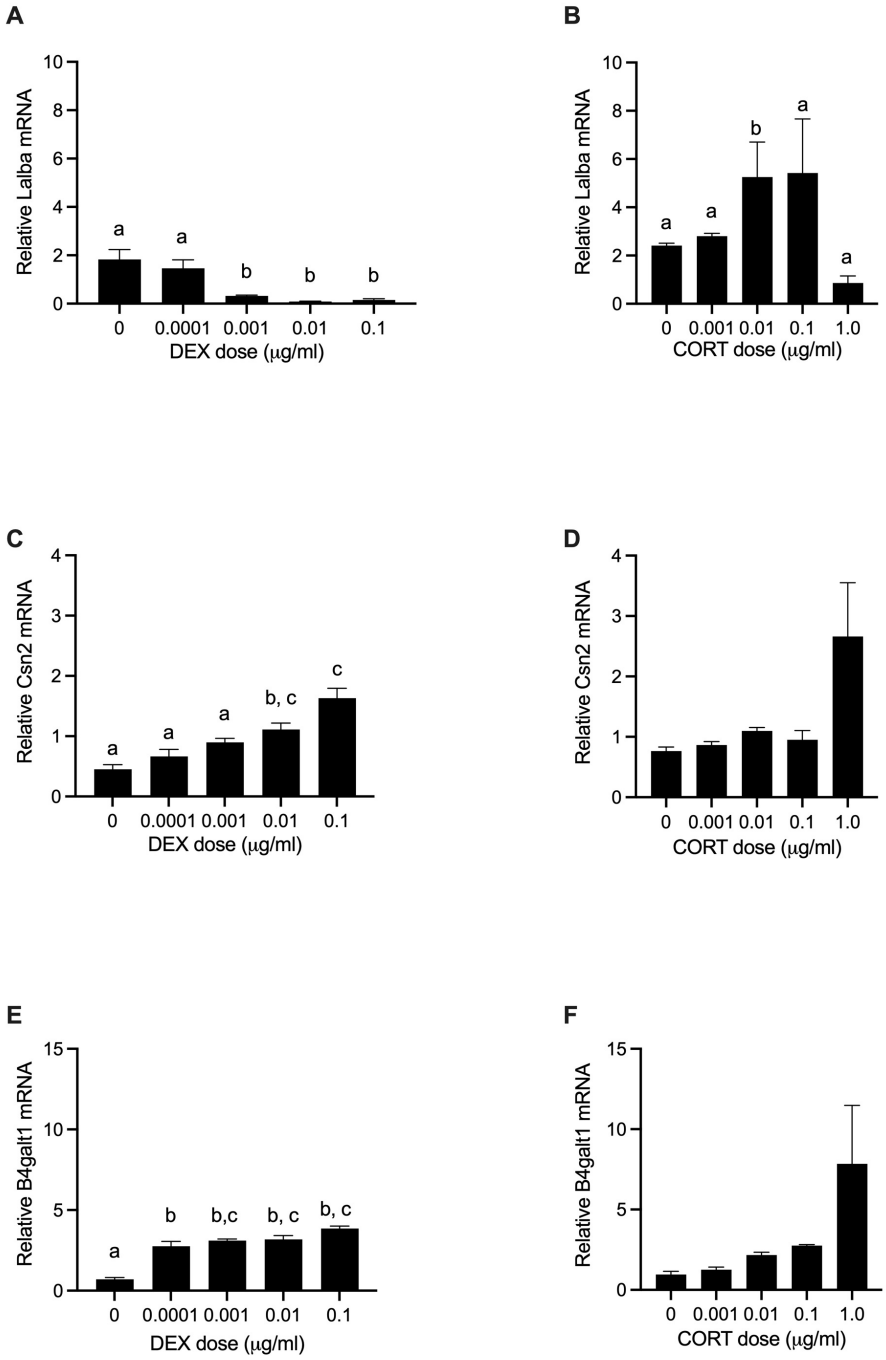

A
Relative Lalba mRNA
10
8
6
4
2
0
a
a
b
b
b
0
0.0001
0.001
0.01
0.1
DEX dose (μg/ml)
B
Relative Lalba mRNA
10
8
6
4
2
0
a
a
b
a
a
0
0.001
0.01
0.1
1.0
CORT dose (μg/ml)
C
Relative Csn2 mRNA
4
3
2
1
0
a
a
a
b, c
c
0
0.0001
0.001
0.01
0.1
DEX dose (μg/ml)
D
Relative Csn2 mRNA
4
3
2
1
0
0
0.001
0.01
0.1
1.0
CORT dose (μg/ml)
E
Relative B4galt1 mRNA
15
10
5
0
a
b
b,c
b, c
b, c
0
0.0001
0.001
0.01
0.1
DEX dose (μg/ml)
F
Relative B4galt1 mRNA
15
10
5
0
0
0.001
0.01
0.1
1.0
CORT dose (μg/ml)

FIGURE 4

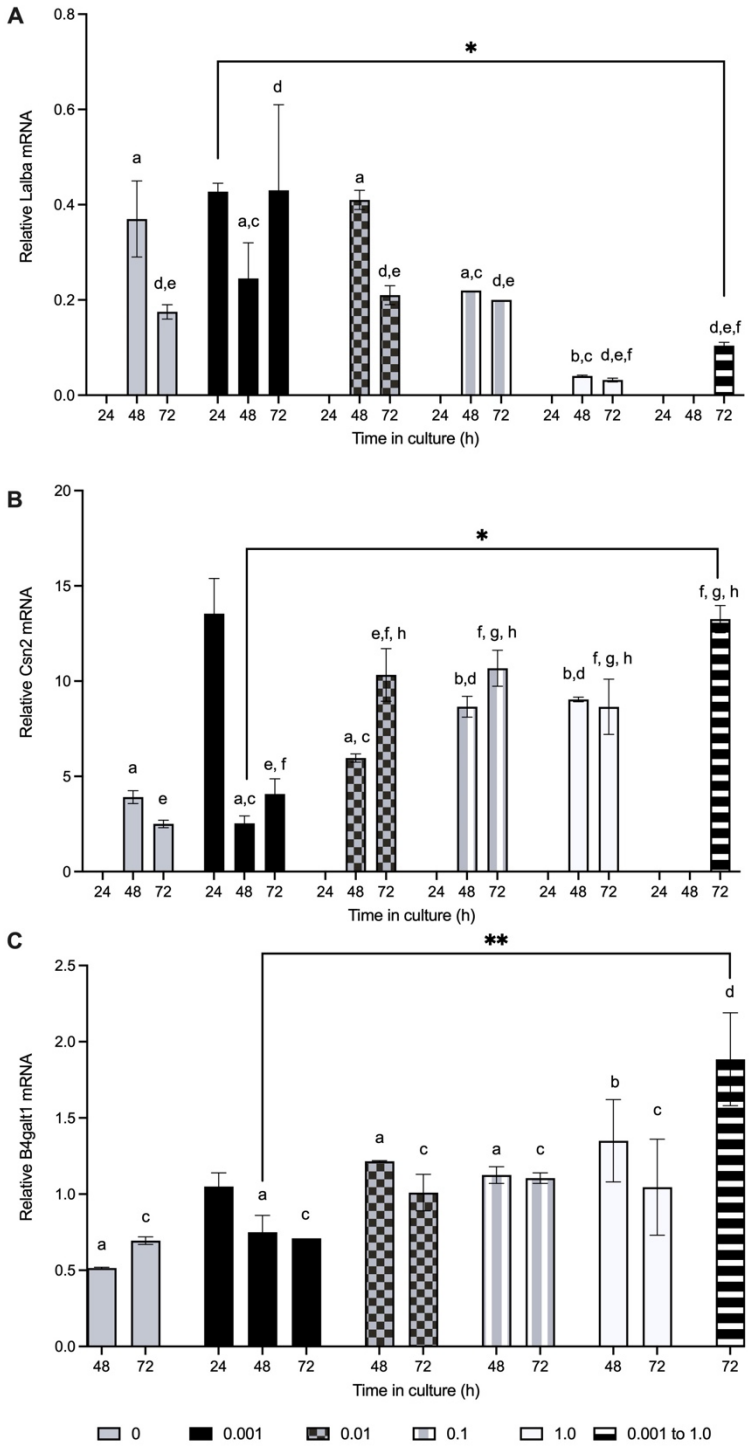

A
Relative Lalba mRNA
0.8
0.6
0.4
0.2
0.0
*
a
d
a
a,c
d,e
d,e
a,c
d,e
b,c
d,e,f
d,e,f
24 48 72 24 48 72 24 48 72 24 48 72 24 48 72 24 48 72
Time in culture (h)
B
Relative Csn2 mRNA
20
15
10
5
0
*
a
e
a,c
e, f
a, c
e,f, h
b,d
f, g, h
b,d
f, g, h
f, g, h
24 48 72 24 48 72 24 48 72 24 48 72 24 48 72 24 48 72
Time in culture (h)
C
Relative B4galt1 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
**
a
c
a
c
a
c
a
c
b
c
d
48 72 24 48 72 48 72 48 72 48 72 72
Time in culture (h)
0
0.001
0.01
0.1
1.0
0.001 to 1.0

FIGURE 5

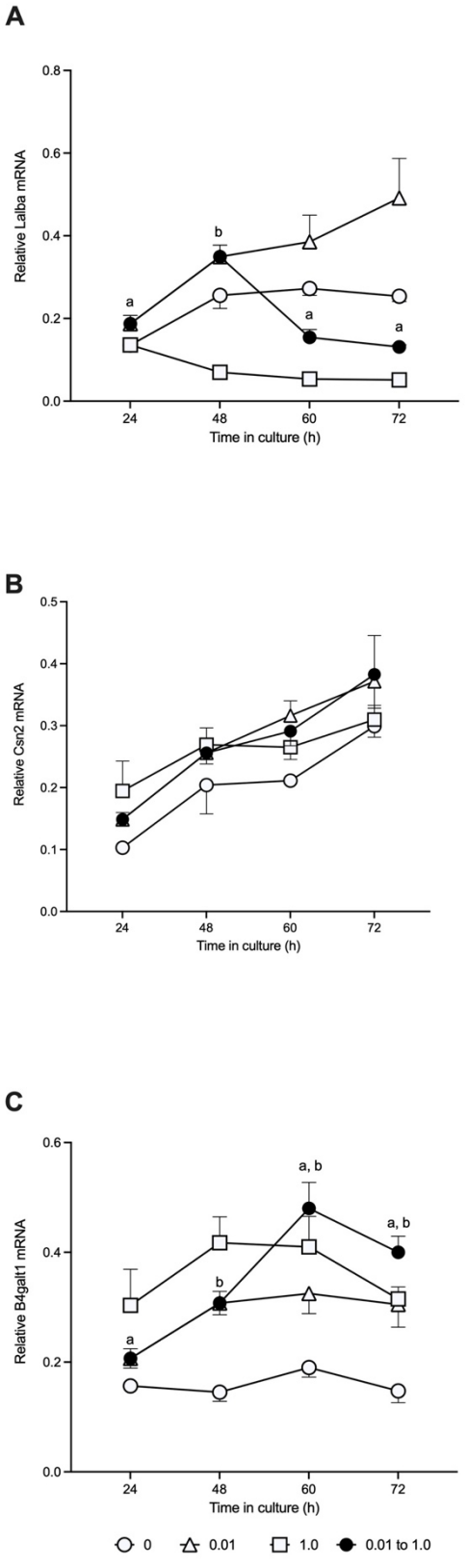

A
Relative Laiba mRNA
0.8
0.6
0.4
0.2
0.0
a
b
a
a
24
48
60
72
Time in culture (h)
B
Relative Csn2 mRNA
0.5
0.4
0.3
0.2
0.1
0.0
24
48
60
72
Time in culture (h)
C
Relative B4galt1 mRNA
0.6
0.4
0.2
0.0
a
b
a, b
a, b
24
48
60
72
Time in culture (h)
0
0.01
1.0
0.01 to 1.0

FIGURE 6

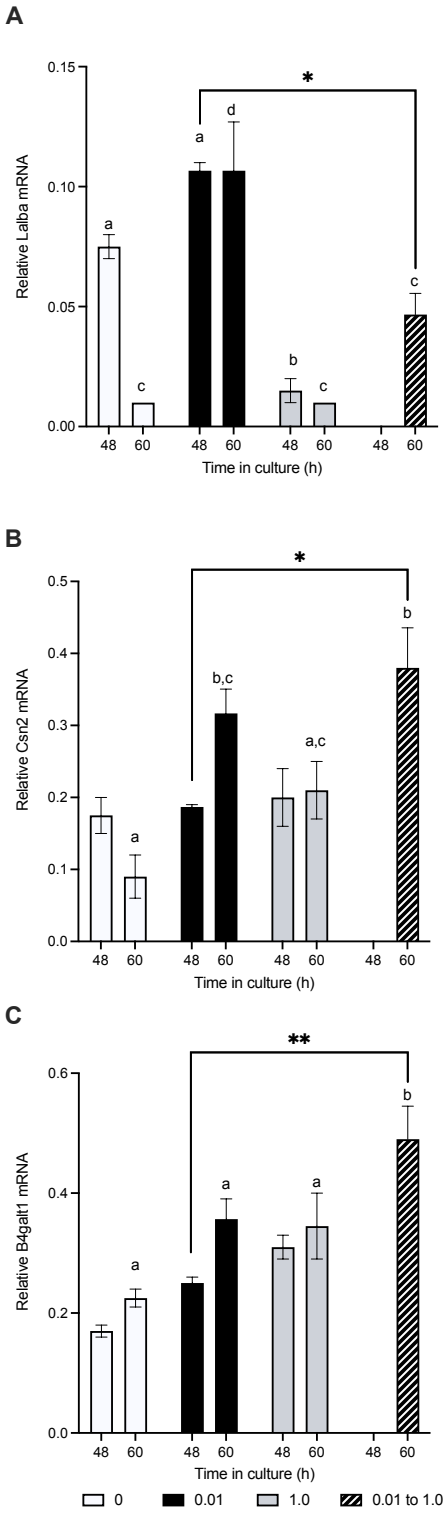
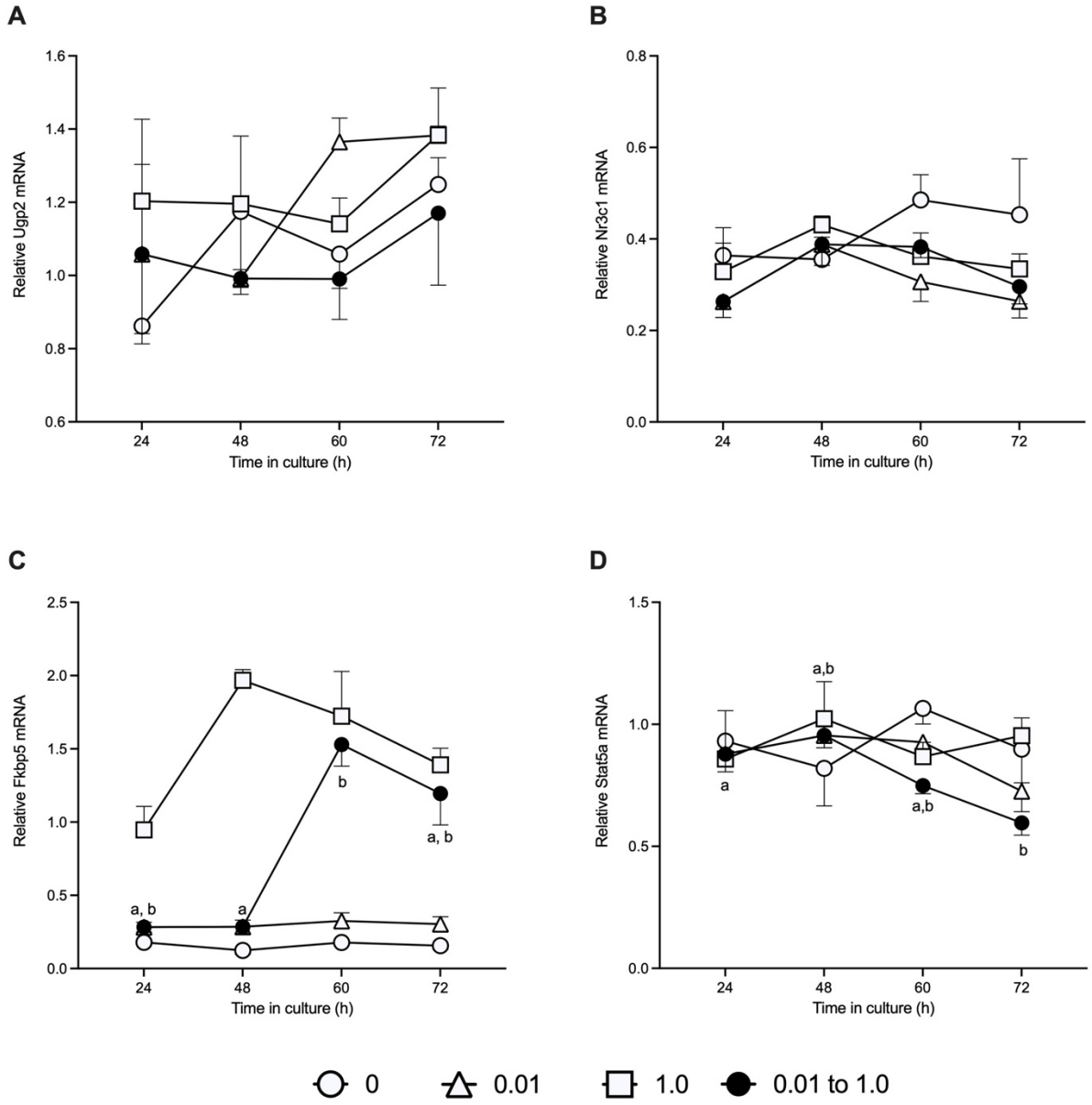

A
Relative Lalba mRNA
0.15
0.10
0.05
0.00
*
a
d
a
c
b
c
c
48 60 48 60 48 60 48 60
Time in culture (h)
B
Relative Csn2 mRNA
0.5
0.4
0.3
0.2
0.1
0.0
*
b
b,c
a,c
a
48 60 48 60 48 60 48 60
Time in culture (h)
C
Relative B4galt1 mRNA
0.6
0.4
0.2
0.0
**
b
a
a
a
48 60 48 60 48 60 48 60
Time in culture (h)
0
0.01
1.0
0.01 to 1.0

FIGURE 7


A
Relative Ugp2 mRNA
1.6
1.4
1.2
1.0
0.8
0.6
24
48
60
72
Time in culture (h)
B
Relative Nr3c1 mRNA
0.8
0.6
0.4
0.2
0.0
24
48
60
72
Time in culture (h)
C
Relative Fkbp5 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
a, b
a
b
a, b
24
48
60
72
Time in culture (h)
D
Relative Stat5a mRNA
1.5
1.0
0.5
0.0
a
a,b
a,b
b
24
48
60
72
Time in culture (h)
0
0.01
1.0
0.01 to 1.0