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RESEARCH ARTICLE

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Patterns of milk macronutrients and bioactive molecules across lactation in a western lowland gorilla (*Gorilla gorilla*) and a Sumatran orangutan (*Pongo abelii*)

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In addition to nutrients, milk contains signaling molecules that influence offspring development. Human milk is similar in nutrient composition to that of apes, but appears to differ in other aspects such as immune function. We examine the longitudinal patterns across lactation of macronutrients, the metabolic hormone adiponectin, the growth factors epidermal growth factor (EGF) and transforming growth factor β2 (TGF-β2), and two receptors for these growth factors (EGF-R and TGF-β2-RIII) in milk samples collected between days 175 and 313 postpartum from a Sumatran orangutan (*Pongo abelii*) and between days 3 and 1,276 from a western lowland gorilla (*Gorilla gorilla*), and compare the results with human data from the literature. Milk macronutrients and hormones were measured using standard nutritional assays and commercially available enzyme immunoassay kits. Ape milk fat content was lower than human milk values, but protein and sugar were similar. Concentrations of all bioactive molecules were consistently detectable except for TGF-β2 in orangutan milk. Concentrations of adiponectin, EGF, and TGF-β2 in both ape milks were lower than found in human breast milk. Concentrations declined with infant age in orangutan milk; in gorilla milk concentrations were high in the first months, and then declined to stable levels until 2–3 years after birth when they increased. However, when expressed on a per energy basis milk constituent values did not differ with age for orangutan and the variation was reduced at all ages in gorilla. In orangutan milk, the ratio of EGF-R to EGF was constant, with EGF-R at 7.7% of EGF; in gorilla milk the EGF-R concentration was 4.4 ± 0.2% of the EGF concentration through 3 years and then increased. These data indicate that potent signaling molecules such as EGF and adiponectin are present in ape milk at physiological concentrations. However, human breast milk on average contains higher concentrations.

KEYWORDS
great ape, growth factor, hormones, milk, nutrition, primate

1 | INTRODUCTION

Milk is the sole food for most mammal neonates during the critical developmental period after birth, and thus must provide the essential nutrients required for early growth and development. However, milk is more than a food and provides much more than nutrients. Milk is a medium through which biochemical messages are transmitted from mother to offspring (Bartol et al., 2008; Koldovský & Štrbák, 1995). Lactation extends the window of direct influence of maternal physiology on the developing neonate and infant, picking up where the placenta leaves off at birth [Bartol et al., 2008; Power & Schulkin, 2013]. For example, human milk contains high concentrations of maternal immunoglobulins (e.g., secretory IgA), which are transferred to the infant and prime the neonatal immune system (Cruz et al., 1982; Hanson et al., 1985; Hanson & Winberg, 1972). Milk also contains physiological levels of growth factors and other hormones (Koldovský,

1994; Koldovský & Štrbák, 1995; Savino, Liguori, Sorrenti, Fissore, & Oggero, 2011), and can exert potent influence on an infant's developing gastrointestinal tract (Lucas & Cole, 1990). Several signaling factors have been shown to influence offspring growth, behavior, health, and reproductive maturation (Anderson et al., 2015; Bartol et al., 2013; Breakey, Hinde, Valeggia, Sinofsky, & Ellison, 2015; Catalani, Alemá, Cinque, Zuena, & Casolini, 2011; Chen et al., 2011; Hinde et al., 2015; Savino, Liguori, Fissore, & Oggero, 2009; Woo et al., 2009).

The comparative study of nursing behavior, age at weaning, and milk composition and transfer can offer insight into the evolution of life histories (Hinde & Milligan, 2011). To date, the most abundant information available for comparative analysis in anthropoid primates is for nutritive factors in milk (e.g., fat, protein, sugar—Hinde, Power, & Oftedal, 2009; Milligan, 2007; Milligan, Gibson, Williams, & Power, 2008; Oftedal & Iverson, 1995; Power, Oftedal, & Tardif, 2002), although there is a growing literature on comparative concentrations of other bioactive factors in anthropoid milk, including those that appear to shape growth, reproductive tissues, gut maturation, metabolism, appetite, and behavior (Anderson et al., 2015; Bernstein & Hinde, 2016; Chen et al., 2011; Donovan & Odle, 1994; Hinde et al., 2015; Woo et al., 2009). In most studies to date, milk bioactives and nutritive factors have been investigated in isolation from one another, but considering nutritional factors alongside other important regulatory molecules is likely to provide the most relevant insight regarding the ultimate function of milk for the infant, and how milk is shaped by maternal physiology. The functions of milk are most appropriately examined from the perspective of regulatory and developmental biology in order to look beyond milk's nutritional importance and to consider milk as a regulatory mechanism crucial for appropriate mammalian development (Hinde & Capitanio, 2010; Nicholas, Simpson, Wilson, Trott, & Shaw, 1997).

We demonstrated that commercially available assays designed to measure two growth factors, epidermal growth factor (EGF) and transforming growth factor β_2 (TGF-β_2), their receptors, and the metabolic hormone adiponectin in human biological samples are effective for measuring these molecules in milk from several old world monkey species and from a Sumatran orangutan (*Pongo abelii*) and a western lowland gorilla (*Gorilla gorilla gorilla*) (Bernstein & Hinde, 2016; Power, Schulkin, Drought, Hinde, & Bernstein, 2013). Prior analyses of these milk components across a diverse array of taxa imply that they are related to maternal traits, and exert important influence on infant somatic, gut, and immune development (Anderson et al., 2015; Donovan & Odle, 1994; Penttila, 2012; Pollack et al., 1987; Woo et al., 2012; Young et al., 1990).

In this paper, we examine the pattern of expression of these bioactive molecules in milk over time during lactation based on longitudinal milk samples collected from these two great apes, and compare the concentration of these milk constituents with published data on human breast milk. We also examine the pattern of macronutrient composition of the ape milks (water, fat, protein, and sugar) to determine if there is any covariance among nutrients and bioactive molecules that might provide either challenges or opportunities for understanding the biological significance of these milk constituents and of any differences among species.

An important technical issue to consider as research on bioactive/signaling molecules in milk proceeds, is what units are the most biologically relevant for expressing and comparing concentrations between individuals and especially between species. The concentrations of milk constituents routinely vary to a certain degree among individuals and even within samples from the same individual. This variation often covaries among multiple milk constituents, and possibly arises from similar causes. For example, the rate of lactose synthesis not only determines the lactose content of the milk, but also affects the osmotic gradient that draws water into the mammary gland, increases milk volume, and dilutes milk constituents (Holt, 1983). Some of the variation in milk constituents is largely explained by variation in water content, and may have little biological significance.

We have previously shown that apparent variation in milk protein content within species and over lactation can be eliminated by expressing protein on a per unit energy basis (Abbondanza, Power, Dickson, & Oftedal, 2013; Power, Verona, Ruiz-Miranda, & Oftedal, 2008). We examine whether expressing the concentrations of other bioactive molecules on a per unit energy basis might also provide insight. We also express our results as ng per mg of milk protein, on the basis that simple volume/water content differences between milk samples would likely affect the concentration of total protein in the same manner as for any individual peptide constituent. If concentration differences between samples were solely due to volume/water content differences, the ratio of the concentration of a peptide to total protein should be largely unaffected. Note that other milk constituents might also serve the same function (e.g., sugar, fat, or mineral content); we chose milk protein since we have found it to be the most consistent, repeatable, and accurately measured major milk constituent, based on the current assay techniques employed at the Nutrition Laboratory of the Smithsonian National Zoological Park, as described in Hood, Voltura, and Oftedal (2009).

2 | METHODS

All research protocols were approved by the Institutional Animal Care and Use committee of the Smithsonian National Zoological Park. The research was conducted in the United States in compliance with all legal requirements and regulations. The research was in compliance with the American Society of Primatologists Principles for the Ethical Treatment of Non Human Primates.

2.1 | Orangutan

Milk samples were obtained from a 40-year-old multiparous female Sumatran orangutan (*Pongo abelii*) living at the Fresno Chaffee Zoo (Fresno, CA) between 175 and 313 days after the birth of her nursing male infant ($n = 16$). Milk was collected by hand expression into 6 ml syringes, vortexed, and then pipetted into labeled 2.5 ml cryovials. Samples were stored in a −80°C freezer and then shipped on dry ice to the Nutrition Laboratory, Smithsonian National Zoological Park (Washington, DC) where they were immediately placed into a −80°C freezer until time of analysis.

2.2 | Gorilla

Weekly milk samples were collected from a multiparous western lowland gorilla (*Gorilla gorilla*) at the Smithsonian National Zoological Park starting from 3 days after the birth of the female infant and continuing into the infant's 5th year of life. The female gorilla had previously been trained to present her chest to the cage mesh in exchange for a food reward as part of standard husbandry training for health assessments. Milk was collected by hand expression into 20 ml cryovials and stored on ice (maximum 2 hr) until it was taken to the Nutrition Laboratory, where it was vortexed, subsampled into smaller cryovials, and stored in a −80°C freezer.

Prior to infant age 556 days, all milk samples were from partial expressions of the mammary gland; starting at day 556 about half of the samples collected were from complete expressions of the mammary gland. The nutrient and hormone content of samples from full and partial expressions between days 500 and 1271 were compared via one-way ANOVA to assess the potential effects on concentrations of a partial versus full expression of the mammary.

2.3 | Assays

All 16 orangutan milk samples were assayed for macronutrient content (protein, fat, and sugar) in addition to adiponectin, EGF, EGF-R, TGF-β_2, and TGF-β-RIII, expressed as ng/ml. Gorilla milk samples from infant age 3 days until 1,053 days ($n = 78$) were assayed for adiponectin, EGF, EGF-R, TGF-β_2, and TGF-β-RIII, expressed as ng/ml. An additional 19 samples from infant age 1,067 days to 1,271 days were assayed only for adiponectin, EGF, and EGF-R. The female gorilla was still lactating beyond 1,271 days. Macronutrient content (milk fat, protein, and sugar) was assayed for 63 of the samples, with milk protein only assayed for an additional five samples from early lactation (infant age 3–25 days).

The protein, fat, and sugar content was used to calculate the gross energy content of the milk (GE), and that value was used to express the values for the EGF, TGF-β_2, and adiponectin on a per energy basis (ng/kcal). The values for EGF, TGF-β_2, and adiponectin were also expressed as ng per mg of milk protein.

2.4 | Nutrient assays

Milk samples were assayed for dry matter (DM), fat, sugar, and crude protein (CP) and gross energy (GE) was calculated from these values. The standard methods utilized have been validated at the Nutrition Laboratory of the Smithsonian's National Zoological Park and performed on milks from about 200 species of mammals (Hood et al., 2009). Briefly, for DM, milk samples were aliquoted, weighed, and dried in a forced air convection drying oven for 3.5 hr at 100°C and then reweighed (AOAC, 1990). Total nitrogen was determined for the milk samples using a carbon, hydrogen, and nitrogen elemental gas analyzer (Model 2400, Perkin Elmer, Norwalk, CT). The obtained total nitrogen value was multiplied by 6.38 to determine the amount of CP in the milk (Jones, 1931). Total lipid was measured using a micro modification of the Roese—Gottlieb procedure (using 150 µl of milk) by means of sequential extractions with diethyl ether, and petroleum ether following disruption of the milk fat globules with ammonium hydroxide and ethyl alcohol (Hood et al., 2009). Milk samples were diluted using distilled water prior to sugar analysis (10 µl of milk into approximately 25 g distilled water). Total sugar was analyzed by the phenol—sulfuric acid colorimetric procedure (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956; Marier & Boulet, 1959) using lactose monohydrate standards. Sugar samples were read at 490 nm either with a UV-visible spectrophotometer (Beckman DU Model 640, Beckman Coulter, Fullerton, CA) with an automatic sipper tube, or with a microplate reader and accompanying software (MRX TC Revelation, Dynex Technologies, Chantilly, VA).

The energy content of milk (GE) was calculated by: GE = 3.95 kcal/g * sugar + 5.86 kcal/g * CP + 9.11 kcal * fat, where sugar, CP, and fat are expressed as g/g (Oftedal, 1984; Perrin, 1958). This formula has been shown to match GE values from bomb calorimetry for rhesus macaque milk (Hinde et al., 2009) and bongo milk (Petzinger et al., 2014).

2.5 | Enzyme-linked immunosorbent assays (ELISA)

Milk samples were assayed for adiponectin, EGF, EGF-R, TGF-β2, and TGF-β-RIII using commercially available assay development kits created for use in human cell culture supernatant. We measured adiponectin using the human Adiponectin DuoSet ELISA Development kit, (R&D Systems, Inc., Minneapolis, MN), EGF using the human EGF DuoSet ELISA Development kit (R&D Systems, Inc.), EGF-R using the human EGF R DuoSet ELISA Development kit (R&D Systems, Inc.), TGF-β2 using the Quantikine Human TGF-β2 Immunoassay kit (R&D Systems, Inc.), and TGF-β-RIII using the human TGF-β-RIII DuoSet ELISA Development kit (R&D Systems, Inc.). We performed parallelism and recovery tests to validate that the component kits were able to accurately measure our analytes of interest. Briefly, we created pools comprised of 15–20 µl aliquots of milk from each individual across the period of lactation represented by the samples. These pools were serially diluted and run in an assay along with the standard curve supplied by the manufacturer. We then tested the parallelism of the slopes of the standard and serially diluted pooled curves (reviewed in Lee et al. (2006)). Since the component kits used were not developed for use with milk, we utilized recovery tests in order to test whether anything in the sample matrix interfered with the assay. To do this, we spiked samples from our species of interest with controls provided in the kit, and measured the % value recovered. Tests of parallelism for all hormones showed very strong ($R^2 \geq 0.98$) correlation between slopes of serially diluted pooled samples. Recovery values from spiking were ≥96%.

Immediately prior to analysis, milk was thawed and gently vortexed, then centrifuged at 4°C. The fat layer was removed and the skim fraction used for analysis. The assays were run in accordance with the manufacturer's protocol and the following modifications. For the adiponectin assay, milk from both species was run at a dilution of 1:3. For the EGF assay, gorilla samples were run at a dilution of 1:300, and orangutan samples at a dilution of 1:80. For the EGF-R assay, gorilla samples were run at 1:10, and orangutan samples at 1:5. Further, the capture antibody was used at 0.5 µg/ml, the detection

antibody used at 150 ng/ml, the reagent diluent included 0.05% Tween 20 in addition to the 1% BSA, and the washing steps included a 30-sec soak. For the TGF-ß RIII assay, gorilla samples were run at 1:40 and orangutan samples at 1:20.

2.6 | Statistical analysis

Concentration values were expressed as both ng/ml and on a per energy basis (ng/kcal) or per mg protein basis. Statistical analyses were performed using IBM SPSS Statistics 20.0 (IBM Corp.©, Armonk, NY). Pearson correlation was used to examine patterns of milk constituent concentration over lactation (infant age). The gorilla milk samples were classified as being from early lactation (birth to 2 months), mid lactation (2–18 months), mid-to-late lactation (18–35 months), and late lactation (after 35 months). Values for measured milk constituents were examined between these time periods by one-way ANOVA with Bonferoni correction. Statistical significance was set at a two-tailed $P < 0.05$.

Unfortunately, published studies that measured EGF, TGF-β2, and adiponectin in human milk generally did not measure nutrients as well, so the values for these hormonal concentrations on a per energy basis in human milk cannot be directly determined. We used a value of 0.675 kcal/g to convert the human milk concentrations to a per energy basis for comparison with the gorilla and orangutan data from this study. This number is the mean value for human milk GE from six different studies from eight different countries summarized in Quinn, Largado, Power, and Kuzawa (2012).

3 | RESULTS

3.1 | Nutrients

Orangutan milk fat and sugar concentrations were variable over this 6-month period of lactation, but unrelated to infant age. Protein concentration was slightly lower than found in milk from humans and other great apes, averaging 0.8 ± 0.1%, and it declined over lactation ($r = -0.572$, $P = 0.021$). However, when expressed on a per energy basis, the relationship between protein and infant age was no longer significant ($r = -0.210$, $P = 0.435$). On a per energy basis, protein content was stable and averaged 15.2 ± 0.8 mg/kcal.

The gorilla milk samples displayed the expected pattern across lactation, with milk protein being high in early lactation (colostrum) starting at around 2.5% but then declining steadily to about 1% by 2 months, after which it was stable until about 18 months (1.05 ± 0.02%; range = 0.91–1.31%). Milk protein increased gradually from 18 months until 35 months and then increased somewhat abruptly to a mean value as high as colostrum after 35 months (Figure 1A; Table 1). Fat content increased ($r = 0.694$, $P < 0.001$) and sugar content decreased ($r = -0.543$, $P < 0.001$) from 2 to 35 months; both decreased after 35 months (Table 1).

When the protein content of gorilla milk is expressed on an energy basis, the pattern changes somewhat (Table 1 and Figure 1B). Protein is still high for colostrum and early milk (before 2 months) and becomes higher and more variable after 3 years of age; however, between 2 and 35 months of age the amount of protein per kcal of milk is quite stable, with little variation between samples ($r = -0.072$, $P = 0.596$; mean = 21.4 ± 0.4 mg/kcal; range = 15.6–27.2 mg/kcal).

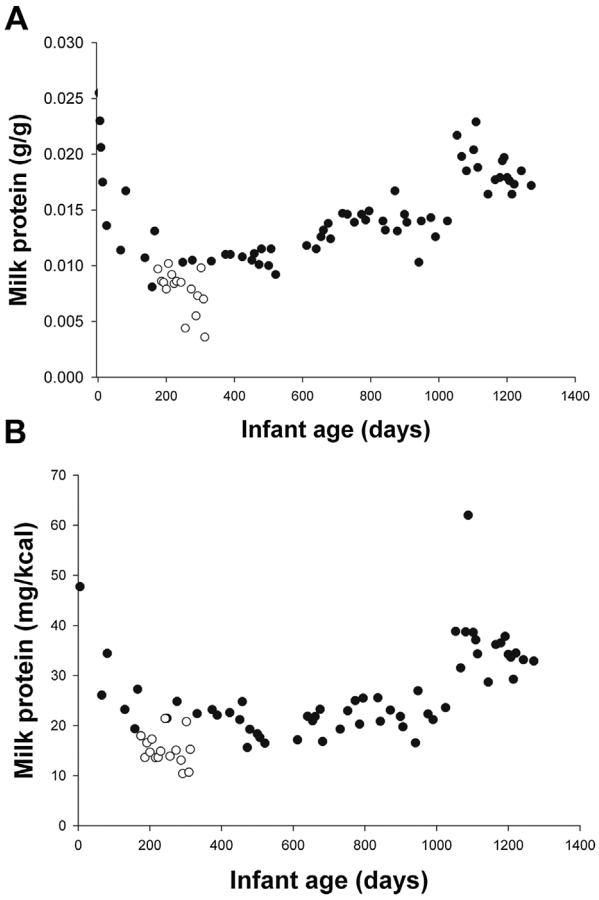

FIGURE 1 Milk protein against infant age. Data for a western lowland gorilla (*Gorilla gorilla*) in filled circles and data for a Sumatran orangutan (*Pongo abelii*) in open circles: (A) milk protein as percent of milk; (B) milk protein per unit of energy (mg/kcal). Between 2 months and 3 years infant age the protein content per unit energy of gorilla milk was relatively constant at 21.3 ± 0.4 mg/kcal. Orangutan milk protein per unit energy was also constant, but at a lower value (15.2 ± 0.8 mg/kcal)

There was no effect of whether the mammary expression was full or partial for any measured gorilla milk constituent, including the bioactive molecules, for samples between days 500 and 1271. Milk fat, the constituent that is most usually assumed to be affected by incomplete mammary gland expression, was, on average, 2.9 ± 0.2% for partial expressions and 3.1 ± 0.2% for full expression ($P = 0.482$).

3.2 | Bioactive molecules

Adiponectin, EGF, EGF-R, and TGF-β-RIII were all detected in orangutan milk; however, TGF-β2 concentration was below the detection limit, suggesting either very low levels of this cytokine in orangutan milk or that the antibody used in the assay is a poor match. The relatively high concentration of its receptor TGF-β-RIII (24.7 ± 1.9 ng/ml) and the presence of TGF-β2 in other primate milks

TABLE 1 Average milk constituent values for one western lowland gorilla (*Gorilla gorilla*) over early, mid, and late lactation

Infant age (days)	DM (%)	CP (%)	Fat (%)	Sugar (%)	GE (kcal/g)	CP (mg/kcal)
3–59	12.4 ± 0.4	1.94 ± 0.15	1.4 ± 0.2	6.4 ± 0.4	.50 ± .02	40.0 ± 7.7
n	7	7	2	2	2	2
66–528	11.0 ± 0.2	1.05 ± 0.02	1.7 ± 0.1	7.1 ± 0.1	.50 ± .01	21.3 ± 0.6
n	29	29	29	29	29	29
549–1,045	12.3 ± 0.2	1.35 ± 0.03	3.1 ± 0.1	6.8 ± 0.1	.63 ± .01	21.5 ± 0.4
n	34	32	34	34	32	32
1,053–1,114	10.4 ± 0.4	1.95 ± 0.11	2.1 ± 0.1	5.9 ± 0.1	.53 ± .02	37.2 ± 1.9
n	19	19	18	18	18	18

n, number of milk samples assayed.

(Bernstein & Hinde, 2016) suggest that it is likely to be present in orangutan milk. The concentration of the two hormones and two hormone receptors measured in orangutan milk declined with infant age when expressed as ng/ml. However, when the concentration was expressed on a per energy basis there was no decline with infant age for any of the four (Table 2).

There was a strong correlation between EGF and its receptor EGF-R in orangutan milk ($r = 0.923$, $P < 0.01$). The ratio of EGF-R to EGF was remarkably constant, averaging 0.077 ± 0.002. Thus, EGF-R has a mean concentration of approximately 7.7% of EGF for these orangutan milk samples between 6 and 12 months of infant age.

In gorilla milk, there was a pattern of high analyte concentration in early infancy (colostrum and early milk) declining to a lower, steady-state concentration until about just past 18 months (for TGFβ2; Figure 2) or 3 years (for adiponectin; Table 3) of infant age, when these analytes displayed increases in concentration. Values for TGF-β-RIII were high for the first 2 weeks and then were stable at a lower level through 3 years (Figure 3). The ratio of TGF-β2 to TGF-β-RIII was highly variable during the period of low TGF-β2 (1 month to 20 months), probably due to those low TGF-β2 values (0.7 ± 0.1 ng/ml). The ratio of these two molecules before 1 month and after 20 months was more stable, but in all cases the concentration of TGF-β2 was lower than that of TGF-β-RIII. EGF was high early in lactation and then was fairly constant afterward through the entire sampling period (Table 3), with a mean of 28.1 ± 0.7 ng/ml. The ratio of EGF receptor to EGF was relatively constant through about 3 years of age (4.4 ± 0.2%), at which point the amount of EGF-R in milk increased (Figure 4). The concentrations of all five analytes were positively correlated with milk protein in gorilla milk across all of lactation and for the four detected analytes in orangutan mature milk (Table 4).

Milk gross energy content (GE) was calculated for the 63 longitudinal milk samples from a single gorilla female that were assayed for fat, protein, and sugar content, and those GE values were used to calculate the concentration of the analytes on a per energy basis (ng/kcal). For the 68 gorilla milk samples with protein content, hormone concentrations were also calculated as ng per mg of protein. Expressing milk adiponectin in these units did not change the pattern across lactation; however, the patterns were different for EGF depending on the units (Table 3). When expressed on a per energy basis, EGF was significantly lower between 18 and 35 months (ANOVA: $P = 0.033$). When expressed as per mg of milk protein, there was no difference between the first 2 months and months 2–18; but ng of EGF per mg protein declined after 18 months (Table 3).

3.3 | Comparisons with human milk

Milk adiponectin concentration generally declined across mid lactation in gorilla (days 66–1,045) and orangutan, similar to humans, although mean adiponectin concentration was low compared to human breast milk (gorilla = 2.1 ± 0.1 ng/ml, orangutan = 1.4 ± 0.1 ng/ml, human = 13.2 ± 2.8 ng/ml; Table 5). Because human milk is consistently higher in fat content (and hence energy) than ape milks so far analyzed, this difference is somewhat ameliorated when hormone concentrations are expressed on a per energy basis (Table 5). The values of

TABLE 2 Correlations between the orangutan infant's age and detectable analytes in orangutan milk, expressed as concentration (ng/ml) and on a per energy basis (ng/kcal)

Concentration	Adiponectin (ng/ml)	EGF (ng/ml)	EGF-R (ng/ml)	TGF-ß-RIII (ng/ml)
Infant age	$r = -0.845$	$r = -0.566$	$r = -0.534$	$r = -0.656$
	$P < 0.001$	$P = 0.022$	$P = 0.033$	$P = 0.006$
Per energy basis	Adiponectin (ng/kcal)	EGF (ng/kcal)	EGF-R (ng/kcal)	TGF-ß-RIII (ng/kcal)
Infant age	$r = -0.395$	$r = -0.035$	$r = -0.025$	$r = -0.339$
	$P = 0.130$	$P = 0.897$	$P = 0.926$	$P = 0.200$

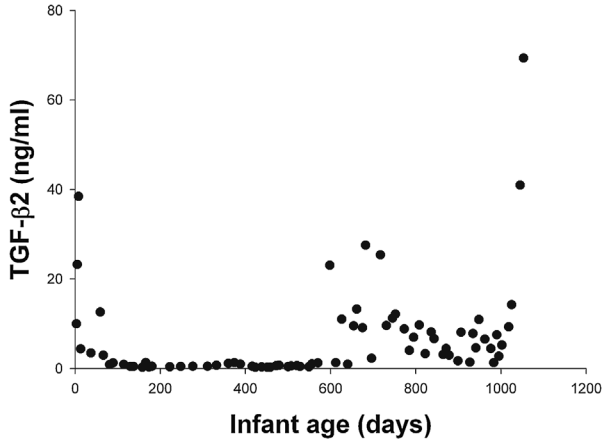

FIGURE 2 TGF-β2 from birth to about 3 years of infant age in milk from a western lowland gorilla (*Gorilla gorilla*)

adiponectin from human milk are still higher, with gorilla milk adiponenctin only 25% of human mean values on an energy basis. Human infants likely are exposed to considerably higher amounts of adiponectin from milk even accounting for a possible increased milk volume consumed by ape infants to achieve the appropriate metabolizable energy intake from milk.

Milk EGF concentrations in this study were highest in gorilla milk (29.7 ± 0.9 ng/ml), followed by orangutan (7.8 ± 0.4 ng/ml); these EGF concentrations are all below those found in mature human milk from western women (75 ± 12 ng/ml; Dvorak, Fituch, Williams, Hurst, & Schanler, 2003), but similar to values found in milk from women in rural Philippines (18.1 ± 0.8 ng/ml; Bernstein & Dominy, 2013). The mid-lactation gorilla milk sample contained low concentrations of TFG-β2 (0.7 ± 0.1 ng/ml) compared to human values from western and rural Philippine women (median of 3.6 ng/ml and mean of 3.2 ± 0.5 ng/ml, respectively).

4 | DISCUSSION

Great ape milk is similar in nutrient composition to human milk, though perhaps lower in fat (Milligan, 2007). The data from this study are consistent with those results, and also show that for gorillas early lactation milk is higher in protein (probably reflecting high levels of immunoglobulins, such as secretory IgA) just as is true for human colostrum. Gorilla milk composition was remarkably stable between 2 and 18 months for all constituents measured. The changes in composition from 18 to 35 months are consistent with reduced nursing frequency leading to reduced lactose synthesis. The changes in composition (higher fat and protein, lower sugar) are consistent with variation in milk composition in other primates and non-primate species that have a more variable composition (e.g., common marmosets, (Power et al., 2008); squirrel monkeys, (Milligan et al., 2008); rhesus macaques, (Hinde et al., 2009); Asian elephants, (Abbondanza et al., 2013)) in which higher fat content is associated with lower sugar content, but with an increase in protein content in proportion to the change in energy density of the milk.

The stability of the protein content of gorilla milk between 2 and 35 months when expressed on an energy basis is an important result. In common marmosets and Asian elephants, where it is also true that protein expressed as mg/kcal is constant over lactation and between mothers with otherwise different fat and sugar milk concentrations, growth is linear (1 g/day for marmosets [Tardif et al., 1998; Tardif, Power, Oftedal, Power, & Layne, 2001]; 900g/day for Asian elephants [Abbondanza et al., 2013]), suggesting that body mass increase of infant gorillas may also be approximately linear over the first 3 years of life. This stability is consistent with data on growth of captive infant gorillas that found relatively constant growth velocities for the first 2–3 years in both male and female infants (Leigh & Shea, 1995). In addition, the amount of protein on an energy basis in milk appears related to the relative rate of growth of infants between species. For example, New World monkeys are considered to have relatively faster growth than Old World anthropoids; and New World monkeys produce milks with a higher protein content on an energy basis (Table 6). Among anthropoids so far studied, humans and orangutans produce the milks with the lowest amount of protein per unit of energy. Among the great apes, gorillas have both the highest milk protein content on an energy basis and the highest growth velocity in the first years of life (Leigh & Shea, 1995, 1996). The growth velocity of human infants in the 1st year is similar to that found for orangutan infants in the first 3 years of life (Heinig, Nommsen, Peerson,

TABLE 3 Average milk concentrations for adiponectin and EGF for one western lowland gorilla (*Gorilla gorilla*) over early, mid, and late lactation expressed in three different units of measure (concentration, energy basis, per mg milk protein)

Infant age (days)	Adiponectin ng/ml	Adiponectin ng/kcal	Adiponectin ng/mg CP	EGF ng/ml	EGF ng/kcal	EGF ng/mg CP
3–59	[a]10.8 ± 1.5	28.6	[a]0.55 ± 0.05	[a]48.2 ± 5.4	127	[a]2.5 ± 0.2
n	7	1	6	7	1	6
66–528	[b]2.6 ± 0.2	[b]5.3 ± 0.6	[b]0.24 ± 0.02	[b]29.8 ± 0.8	[a]58.9 ± 2.6	[a]2.7 ± 0.1
n	29	18	18	29	18	18
549–1,045	[b]2.9 ± 0.1	[b]4.7 ± 0.2	[b]0.22 ± 0.01	[b]25.9 ± 0.7	[b]42.0 ± 1.7	[b]2.0 ± 0.1
n	41	28	28	41	28	28
1,053–1,114	[a]10.2 ± 1.4	[a]21.6 ± 4.2	[a]0.56 ± 0.09	[b]30.2 ± 2.4	[a]60.0 ± 5.5	[b]1.7 ±.1
n	20	17	18	20	17	18

n, number of milk samples assayed.
Values with different superscripts within a column are significantly different (one-way ANOVA with Bonferoni correction, $P < 0.05$).

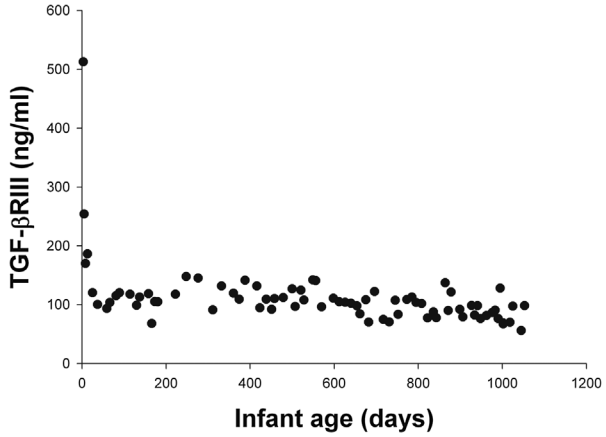

FIGURE 3 TGF-βRIII from birth to about 3 years of infant age in milk from a western lowland gorilla (*Gorilla gorilla*)

Lönnerdal, & Dewey, 1993; Leigh & Shea, 1995). Interestingly, the lower protein per unit of energy is accomplished in human milk by increasing milk energy density through an increase in milk fat, while in orangutan milk it is achieved by lowering protein concentration.

After about 3 years of age for the gorilla infant, the milk produced by its mother changed in composition, with protein increasing, and fat and sugar values decreasing (Table 1). It is not certain how much milk the infant was consuming at this age, as the keeper staff did not report any observations of nursing behavior after 2 years of age; however, the female continued to lactate and provide sizeable milk samples. In the wild, the infant would probably still be nursing; wild western lowland gorilla infants are thought to be weaned between 4 and 5 years of age (median 4 years, 9 months; Breuer, Breuer-Ndoundou Hockemba, Olejniczak, Parnell, & Stokes, 2009). In a captive setting with abundant, easy to consume and digest food, this infant may have reduced its milk intake to low amounts by 3 years of age, perhaps only nursing during the night. The higher variability among all the milk constituents may not represent the normal pattern of late lactation in wild gorillas; but rather may reflect normal changes in mammary gland synthesis associated with involution due to reduced suckling stimulation.

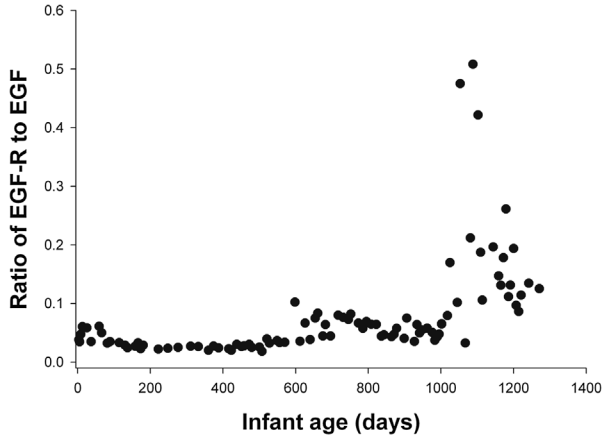

FIGURE 4 The ratio of the EGF receptor to EGF from birth to about 4 years of infant age in milk from a western lowland gorilla

TABLE 4 Analyte concentrations correlated against milk protein levels in gorilla and orangutan milk

	Orangutan	Gorilla
Adiponectin	$r = 0.589$ ($P = 0.016$)	$r = 0.659$ ($P < 0.001$)
EGF	$r = 0.526$ ($P = 0.036$)	$r = 0.469$ ($P < 0.001$)
EGF-R	$r = 0.416$ ($P = 0.109$)	$r = 0.572$ ($P < 0.001$)
TGF-β2	ND	$r = 0.629$ ($P < 0.001$)
TGF-βRIII	$r = 0.600$ ($P = 0.014$)	$r = 0.567$ ($P < 0.001$)

ND, not detected.

This study confirms that, similar to human milk, milks from two of our closest relatives contain significant concentrations of biologically important hormones, such as EGF, TGF-β2, and adiponectin, at physiological concentrations for an extended time during lactation. The stability of the concentrations of the measured bioactive factors over an extended time during established lactation, years in the case of the gorilla, indicates that maternal signaling via milk may be important well beyond neonatal life, and into later infancy.

The detection of the two receptors in milk raises interesting questions. The stability of the ratio of EGF-R to EGF in both orangutan and gorilla mid lactation milk samples suggests a strong connection between the expression of these two genes by the mammary gland (though possibly differences between the species). What role EGF-R may be playing is uncertain. It may be acting as a binding protein, either regulating the action of EGF or possibly providing protection of EGF from stomach acids and digestive enzymes. EGF and EGF-R may be acting as a complex to produce signaling by binding onto infant intestinal epithelial cells, a process known as trans-signaling, where a soluble receptor-ligand complex binds to cells to allow signaling even in the absence of a cellular membrane-bound receptor. This is known to occur for IL-6 and its soluble receptor sIL-6R (Rose-John, Scheller, Elson, & Jones, 2006).

Although we cannot be certain, the form of TGF-β-RIII we measured is probably the soluble form. This form of TGF-β-RIII has been found in rat milk, and mother-nursed rat pups had higher levels of TGF-β-RIII staining in intestinal epithelium compared to rat pups fed an artificial formula containing no TGF-β-RIII (Zhang, Zola, Read, & Penttila, 2001). TGF-β-RIII binds all isoforms of TGF-β. Immunoprecipitation of TGF-β-RIII from rat milk decreased TGF-β activity (TGF-β2 is the dominant isoform in rat milk), implying that TGF-β-RIII was binding active TGF-β (Zhang et al., 2001). Although TGF-β-RIII appears to lack a signaling motif, it has been shown to be required for TGF-β signaling in some circumstances (e.g., Brown, Boyer, Runyan, & Barnett, 1999), possibly acting in concert with TGF-β-RI and/or TGF-β-RII. The concentration of TGF-β-RIII in orangutan and gorilla milk appears to be much higher than the concentration of TGF-β isoforms (assuming TGF-β2 is the dominant isoform in milk), suggesting TGF-β-RIII may have biological roles besides regulation of TGF-β signaling.

Lactation as a reproductive adaptation has fundamentally affected mammalian biology. It allowed the dramatic reduction of maternal resources deposited into the egg (Brawand, Wahli, & Kaessmann, 2008). All the necessary biochemical basis for life were no longer

TABLE 5 Values for adiponectin in mid lactation milk for human (from the literature, with estimated values on an energy basis) and for gorilla and orangutan from this study

Source	Concentration (ng/ml)	*Energy basis (ng/kcal)
Weyermann, Rothenbacher, and Brenner (2006)	12.8 ± 0.4	19.0 ± 0.5
Woo et al. (2009)	20.3 ± 0.4	30.1 ± 0.6
Bronsky et al. (2011)	21.7 ± 0.5	32.1 ± 0.7
Ley et al. (2012)	12.3 (median)	18.2
Bernstein and Dominy (2013)	4.3 ± 0.1	6.4 ± 0.1
Anderson et al. (2015)	7.5 ± 5.8	11.1 ± 8.6
Mean values for human milk	13.2 ± 2.8	19.5 ± 4.1
Gorilla milk this study (days 66–1045)	2.8 ± 0.1 (21% of human mean)	4.9 ± 0.3 (25% of human mean)
Orangutan milk this study	1.4 ± 0.1 (11% of human mean)	2.7 ± 0.2 (14% of human mean)

*Assuming a mean human milk GE of 0.675 kcal/g.

needed to be included, as lactation allowed an extended time period to transfer maternal resources to offspring. The advent of the placenta further enhanced this developmental strategy by allowing the direct transfer of gases, nutrients, and bioactive molecules from maternal circulation. After birth, this intimate transfer of maternal resources to her offspring continues via milk. In a placental mammal, maternal biochemical signaling which guides and enables fetal and neonatal development is a continuous process over an extended period of maternal dependence (Bartol et al., 2008; Power & Schulkin, 2013). The normal development of mammalian fetuses and neonates depends upon these maternal signals (Hinde et al., 2015). These signals change over time, not only in a programmed way that matches the changing developmental circumstances as the offspring ages and matures, but also as maternal circumstances change. Mammalian mothers may be able to alter their developmental signals depending on the maternal environment, or in some cases may have no choice, as the signaling depends upon maternal circumstances.

Not surprisingly, maternal signaling pre- and postpartum often shows continuity. For example, two of the molecules we measured in milk (EGF and TGF-β2) are found in human amniotic fluid, which is swallowed by the developing fetus (Lang & Searle, 1994; Underwood, Gilbert, & Sherman, 2005). Thus, in utero the developing gut is exposed to these (and other molecules) of maternal origin which likely have regulatory and developmental function and are likely necessary for the appropriate development of the gut. After birth the signaling continues, now via milk. It is instructive to consider that milk was the evolutionary original source of most potential maternal signals in early mammals. The delivery of maternal signals to offspring orally likely is an ancient adaptation in mammals. After the evolution of the chorio-allantoic placenta, with the fetus enclosed within the amniotic sac for a much longer period of development, fetal swallowing of amniotic fluid allowed this signaling to continue. The adaptation of lactation may have served as a preadaptation to signaling via amniotic fluid (Power & Schulkin, 2013).

TABLE 6 Milk protein content for anthropoid primates on a g/g basis (%) and a mg/kcal basis

Genus	Milk protein (%)	Milk protein (mg/kcal)	Source
New World monkeys			
Alouatta	2.25	40.0	Milligan (2007)
Saimiri	3.6	39.4	Milligan et al. (2008)
Cebuella	2.9	35.6	Power et al. (2002)
Leontopithecus	2.3	35.4	Power et al. (2002)
Callithrix	2.45	34.1	Power et al. (2002, 2008)
Aotus	2.8	31.1	Power, unpublished data
Cebus	2.4	27.0	Milligan (2009)
Old World monkeys			
Macaca	1.95	21.6	Hinde et al. (2009)
Papio	1.5	18.4	Power, unpublished data
Hominoids			
Gorilla	1.4	26.7	Whittier et al. (2011) (mountain gorilla)
Gorilla	1.2	21.3	This study (days 66–1,045)
Pan	0.9	17.1	Milligan (2007)
Homo	1.04	15.2	Quinn et al. (2012)
Pongo	0.78	15.2	This study

4.1 | Units of measure

In urine, hormone levels are rarely if ever expressed as a straight concentration as measured. Usually, hormone concentration is adjusted to creatinine, in part to account for the large potential variation in water content of urine. Milk is a regulated fluid; but it does not appear to be as tightly regulated as blood, though more regulated than urine. Cortisol concentration in milk has been shown to vary with milk production (Hinde et al., 2015). Unfortunately, there is no obvious milk constituent to serve the function of creatinine for urine. Rather than comparing absolute amounts, the relative proportions of milk constituents, including hormones, may better reflect the biological significance of differences between samples, individuals, and species.

The total amount of hormone (or any other bioactive factor, such as a nutrient or immunoglobulin) transferred per day via milk depends upon the concentration in milk, but also on the volume of milk consumed. Although the factors that regulate milk intake by infants have not been fully determined, it is reasonable to propose that in early infancy, milk intake must satisfy energy requirements. Infants consuming a low-energy milk, such as the orangutan milk in this study, are likely to ingest a larger volume of milk to meet their basic needs. Comparing concentrations of any milk constituent between different species, or even within a species, can be misleading if the energy content of the milks vary. A lower concentration of a milk constituent may not indicate that an infant receives less, as the infant may regulate his intake and/or the mother may regulate milk production to enable a similar total amount of the constituent to be ingested despite differing concentrations.

We propose two potential units for expressing hormones in milk that might enable better comparisons between samples, especially for comparisons across species: expressing levels on a per unit of energy basis (e.g., units of ng/kcal), and expressing levels per mg of milk protein (units ng/mg). The patterns of some gorilla milk constituents measured in this study differed depending upon the units of measure, suggesting that these different units may be telling us different stories. The challenge is to determine which pattern (units) best describes the phenomenon of interest and thus reflects most accurately the biological significance of any variation or lack thereof.

It is disappointing that the literature on human milk bioactive factors usually does not include measures of any of the macronutrients. One study on human milk adiponectin did measure total protein and showed that it was correlated with adiponectin (Ley, Hanley, Sermer, Zinman, & O'Connor, 2012). As evidenced from the results of this study, the concentrations of milk constituents often covary. We suggest that the interpretation of variation in the concentration of any milk constituent benefits from knowledge of the concentration of macronutrients in milk.

4.2 | Human versus ape milk

Human studies indicate that on average, the concentrations of the measured hormones in human milk are higher than were found in this study of ape milks, even after accounting for the slightly higher energy content of human milk (Table 5). More data from more individuals are needed before we can consider ape milks to be appropriately characterized, especially in light of the large variation in concentrations of these hormones in milks from different human populations. The range of concentrations found in different human populations indicates that many factors both internal and external to the mother likely affect the concentrations of these hormones in her milk. The fact that women from two Filipino populations have lower milk concentrations of adiponectin and EGF (but not TGF-β2) compared to western women raises the possibility that the values we are finding in modern western women for some bioactive factors may be outside of the evolutionary norm for humans in the past, or at least that our conception of "normal" levels based on well-nourished populations does not capture the full range of adaptive physiological variation in humans (Anderson et al., 2015). At least for EGF, the concentrations in milk are not different between captive gorillas and orangutans and women from rural Philippines whose stature, body condition, nutrition and disease history, and so forth differ markedly from western women. Of course captive great apes may be more analogous to western women, as the captive environment provides more food, less disease and other challenges than these apes experience in the wild. We hypothesize that the concentrations of these hormones in wild gorilla and orangutan milk may be lower than we found in our captive specimens, consistent with the human data. This is an empirical question that could be answered, though not without great difficulty.

Assuming human milk does, in general, contain higher amounts of these bioactive factors, the implications are uncertain at this time, though hypotheses can be suggested. Aspects of human development may be more rapid than in apes, driven by enhanced maternal signaling through milk. For example, higher concentrations of growth factors important for intestinal development (EGF and TGF-β2) may relate to a faster development of the human infant gut, allowing earlier introduction of supplemental foods. The adiponectin receptor 1 (AdipR1) is found in high concentrations in the small intestine of mouse embryos, and binding by adiponectin is suggested to improve gut barrier function (Zhou et al., 2005). The ability to feed infants supplemental foods at an early age (due to the advent of cooking, other means of food processing, domestication of grains, and so forth) could have had profound effects on the survival and fecundity of mothers as well as their children by providing an alternative nutritional resource for infants (Kennedy, 2005). But supplemental foods (especially those of long ago) are not as easily digestible as breast milk, and early supplementation might have produced selective pressures to speed up intestinal maturation.

The strengths of this study are the longitudinal nature of the samples, especially for the gorilla, and the measurement of macronutrients as well as bioactive molecules allowing the investigation of the potential that changes in one milk constituent may be associated with changes in others. The obvious weakness is that these results apply to a single individual during a single lactation event for each species. The nutrient data are consistent with the few other published data on these species (Milligan, 2007) and the mountain gorilla (Whittier, Milligan, Nutter, Cranfield, & Power, 2011). There are no other published data on the hormones/growth factors measured in this study for these species. The evidence from the human data suggest that the hormone values may not be representative of all

orangutan and gorilla milk, especially between milk from wild and captive individuals. On balance, the results support the hypothesis that there are species differences in the concentrations of milk bioactive factors that likely result in different levels of signaling via milk between humans and great apes, but more data on apes and from different human populations are needed to assess the hypothesis further.

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