

# Milk at Altitude: Human Milk Macronutrient Composition in a High-Altitude Adapted Population of Tibetans

Elizabeth A. Quinn,<sup>1\*</sup> Kesang Diki Bista,<sup>2</sup> and Geoff Childs<sup>1</sup>

<sup>1</sup>Department of Anthropology, Washington University in St. Louis, St. Louis, Missouri 63130

<sup>2</sup>Institute of Medicine, Tribhuvan University, Kathmandu, Nepal

**KEY WORDS** lactation; high altitude; milk composition; breast milk; infants

## ABSTRACT

**Objective:** The physiological challenges of high altitude have led to population-specific patterns of adaptation. These include alterations to child growth and reproduction, including lactation. However, while breastfeeding has been investigated, nothing is known about milk composition in high altitude adapted populations.

**Materials and Methods:** Here, we investigate milk macronutrient composition, volume, and energy in a sample of 82 Tibetans living at high and low altitude in rural villages (Nubri Valley, Nepal) and at low altitude in Kathmandu, Nepal. Milk samples were collected in the morning using hand expression, frozen, and assayed for fat, protein, and total sugars. Reproductive histories and health recalls were also collected.

**Results:** Milk fat averaged  $5.2 \pm 2.0$  g/100 mL, milk sugar  $7.37 \pm 0.49$  g/100 mL, and milk protein  $1.26 \pm 0.35$  g/100 mL for a mean energy density of  $81.4 \pm 17.4$  kcal/100 mL. There were no associations between altitude of residence and milk composition; however, overall milk fat was high compared to reference populations. Within the three groups, milk fat was positively associated with infant age ( $B = 0.103$ ;  $p < 0.001$ ) and maternal triceps skinfold thickness ( $B = 0.095$ ;  $p < 0.01$ ) while milk sugar was significantly and inversely associated with maternal parity and triceps skinfold thickness.

**Discussion:** Milk fat, and consequently milk energy, may be increased in high-altitude adapted Tibetans when compared to populations living at low altitude. The association between milk fat and maternal adiposity suggests that milk composition may be sensitive to maternal adiposity in this sample, likely reflecting increased metabolic costs of producing a high-fat milk. *Am J Phys Anthropol* 159:233–243, 2016. © 2015 Wiley Periodicals, Inc.

The physiological stressors of living at high altitude have been well described elsewhere (Beall, 2013; Bigham et al., 2013; Moore et al., 2000; Moore et al., 1998). Among high-altitude adapted populations living in the Andes, Ethiopian Highlands, and Tibetan Plateau, numerous genetic and phenotypic adaptations have been selected for facilitating functioning under the specialized ecological pressures associated with high altitude (Alkorta-Aranburu et al., 2012; Beall, 2000; Beall, 2006; Moore et al., 2006). It has been hypothesized that such pressures should be most acute during early life because of immature physiology, thus making infancy a period of intense selection on environmentally appropriate phenotypes (Kuzawa and Quinn, 2009; Leonard, et al., 2002; Wells, 2012) and genotypes (Moore, 2001).

Many of the ecological pressures hypothesized to influence reproduction, including lactation, are well documented in high-altitude adapted populations (Vitzthum, 2001). However, while there has been research into reproductive health, child growth, and breastfeeding behaviors among populations from the Andes and Himalayas, there has been no study integrating milk composition as a part of this biological variation. Here, we investigate the associations between milk volume, energy, and macronutrient composition and high-altitude adaptation, looking at three groups of ethnic Tibetans: high- and low-altitude mothers living in the rural Nubri Valley, Nepal and low-altitude mothers living in urban Kathmandu, Nepal.

We hypothesized that there would be a dose-dependent association between altitude of maternal resi-

dence and the amount of fat in a woman's milk (and consequently energy). As an alternative hypothesis, we predicted that altitude may have no association with milk volume, energy, or macronutrient composition; instead secondary predictors associated with maternal and infant characteristics would be the primary drivers of individual variation in milk composition. In this second model, mothers are somatically buffering milk composition and volume, with possible long-term consequences for maternal health. Maternal adiposity, we predict, would be positively associated with milk volume, fat, and energy.

In many of the prior studies conducted in both well- and under-nourished populations living below 3000 m, the macronutrient composition of human milk is comparably buffered from nutritional shortfalls (Prentice et al.,

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\*Correspondence to: Elizabeth Quinn; Department of Anthropology, Washington University in St. Louis, One Brookings Drive, Campus Box 1114, Saint Louis, MO 63130, USA. E-mail: equinn@wustl.edu

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1994; Villalpando and Del Prado, 1999; Brown et al., 1986).

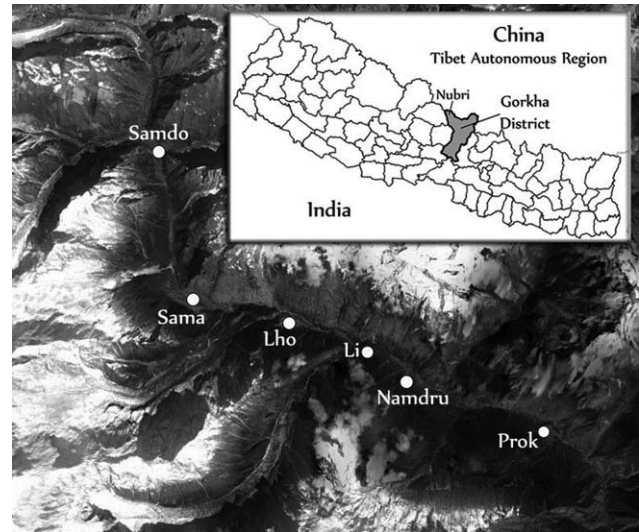
Among humans, maternal milk synthesis is adequate even under conditions of severe malnutrition, although volume may be compromised (Jelliffe and Jelliffe, 1978). Maternal somatic buffering appears to only apply to milk macronutrients and to a lesser extent volume; there is considerable documented global variation in milk fatty acids, micronutrients, hormones, and immune factors reflecting dietary or ecological pressures (Bernstein and Dominy, 2013; Butte et al., 1988; Insull et al., 1959; Ley et al., 2012; Miller and McConnell, 2015; Prentice, 1995; Quinn et al., 2015; Santos et al., 2013; Savino and Liguori, 2008; Smit et al., 2002).

Evidence for the importance of maternal fat reserves on milk composition has been well documented (Brown et al., 1986; Butte et al., 1984; Prentice et al., 1994; Villalpando and del Prado, 1999; Villalpando et al., 1992). These associations are largely concentrated in marginally or under-nourished populations. Several studies have reported positive associations between measures of maternal adiposity and milk volume and fat: Otomi women living in rural Mexico, body fat was a primary predictor of milk volume and milk fat (Villalpando et al., 1992); both Prentice et al., (1981) and Brown et al., (1986) have reported positive associations between maternal triceps skinfold thicknesses and milk volume and fat in women from the Gambia and Bangladesh, respectively.

During pregnancy, adipose tissue is preferentially deposited in the trunk and thighs, areas that show the greatest evidence for fat mobilization in both well- and under-nourished populations (Butte et al., 1984; Butte and Hopkinson, 1998; Sohlström and Forsum, 1995), with the triceps having comparatively minor importance (Sohlström and Forsum, 1995). In well-nourished populations, triceps skinfold thicknesses may even increase during lactation, despite concurrent fat losses in the trunk and thighs (Butte et al., 1997; Nommsen et al., 1991).

For these and other reasons, humans have been considered to be both income and capital breeders by prior studies (Van Noordwijk et al., 2013). Income breeders, with regards to lactation, have milk (and other aspects of reproduction) dependent on maternal food (Stephens et al., 2009). The classic example is the rat, where maternal malnutrition predicts both decreased milk volume and composition (Pine et al., 1994). Capital breeders, on the other hand, rely primarily on somatic resources to support reproduction and lactation. For milk production, one of the best examples is the polar bear, who will give birth during hibernation and initially lactate with minimal food intake (Derocher et al., 1993).

Here, we consider humans to be intermediate capital breeders. In humans, Vernon and Pond (1997) observed that “well-fed” humans have limited need to draw on food reserves”. Considerable evidence shows that human milk composition is relatively well buffered across maternal condition, although volume may decline with severe maternal malnutrition. Moreover, “well fed” is not defined in the original publication, and may only refer to a small subset of individuals—primarily contemporary Westerners. Income breeding may not, as others have suggested, be a species-typical characteristic for humans. In humans, it appears that dietary energy is preferentially utilized; however, body fat can be employed as necessary and accumulation of body fat dur-



**Fig. 1.** Map of the Nubri Valley showing the location of the six study villages. Namrung and Namdru are alternative spellings for the same village. The insert illustrates the location of Nubri within Nepal.

ing pregnancy is well-established for human pregnancies. Dewey (1998), for example, has cautioned that “mobilization of body fat is a characteristic of lactation, but not a universal”; even in “well-fed” populations, declines in adiposity over the course of lactation are not uncommon (Butte and Hopkinson, 1998; Butte et al., 1997; Butte et al., 1999; da Silva et al., 2013). In populations with a high degree of food insecurity or seasonal fluctuations in nutritional intakes, adipose tissue may be an important energy reservoir for lactation.

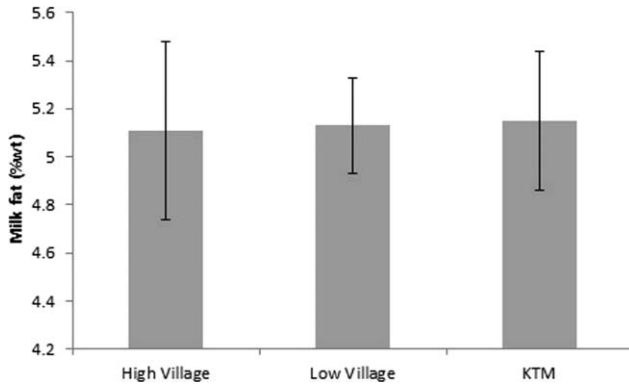
In fact, such emphasis on income breeding may in fact be relatively recent—and evolutionarily novel—are superimposed on physiology that anticipates using adipose stores at least intermediately during lactation. Jönsson (1997) predicted that in highly seasonal environments where metabolic demands or food intake could vary widely, capital breeders might overinvest in somatic reserves. We might therefore predict that in highly stressful environments or environments characterized by a high degree of seasonality and/or limited nutrition, maternal adipose tissue may be an important energy reserve for supporting the metabolic costs of lactation.

## METHODOLOGY

### Populations and field sites

**High- and low-altitude rural—Nubri Valley, Nepal.** The Nubri Valley is an ethnically Tibetan enclave situated in the highlands of Gorkha District, Nepal, part of the Himalayas (Fig. 1). Village altitudes range from 6,900 to 12,570 ft (2,090–3,830 m) and are populated by roughly 3,000 inhabitants. For the purposes of this study, villages were stratified into high and low categories, following prior research in the Nubri Valley (Beall and Leslie, 2014), with high villages defined as those above 10,000 ft in altitude and low villages defined as those below 10,000 ft in altitude.

Nubri’s inhabitants are descendants of migrants from the Tibetan Plateau and southern lower valleys. Occupation histories date back at least 700 years ago, with trans-Himalayan migration continuing until recently



**Fig. 2.** Mean milk-fat comparison between Nubri mothers living at high (>10,000 ft) and low (<10,000 ft) altitude and control mothers living at low altitude in Kathmandu. Models were adjusted for maternal parity, infant age, and maternal triceps skinfold thickness. There were no significant differences.

(Childs, 2004b). Nubri residents subsist on agriculture (barley, potatoes, maize), animal husbandry (yaks and yak-cow crossbreeds), trade (timber, medicinal plants), and a growing tourism and mountaineering industry (Childs, 2004b). The valley has been marginalized from economic development; educational, and health services remain rudimentary.

Forty-one mothers from six villages were recruited from the Nubri Valley for this study, with 26 living at high altitude (>10,000 ft) and 15 living at low altitude (<10,000 ft). Infant mortality rates among the low-altitude sample were higher, limiting the number of available mother-infant dyads for the study.

**Low-altitude urban—Kathmandu, Nepal.** A comparison sample of 41 ethnic Tibetan women living at low altitude was recruited from Tibetan communities in Kathmandu, Nepal. Kathmandu, the capital of Nepal, sits at an altitude of 4600 ft (1400 m) above sea level, and has a population of roughly 1 million. While technically not “low altitude”, Kathmandu is at a much lower altitude than the two rural samples and also provides an urban dimension to such comparisons. Kathmandu is also home to the largest population of Tibetans outside of Tibet, making it an ideal place to recruit an urban, lower altitude sample. Kathmandu is also the primary migration loci for individuals from the Nubri Valley, which allowed for inclusion of women born in Nubri but now living elsewhere in the study.

Mothers were recruited from neighborhood mothers or women’s groups associated with Tibetan refugee settlements, NGOs, day care centers, and social networks (for example, women born in Nubri but now living in Kathmandu). While this may introduced some bias into the Kathmandu sample, it represented the best strategy for recruitment in a large urban setting like Kathmandu, where the Tibetan population is dispersed making door-to-door recruitment virtually impossible.

Mothers varied in the amount of time they had spent at low altitude—a few had moved to Kathmandu as young children or adults (either from Nepal or Tibet;  $n = 3$ ) while the majority were born in Kathmandu. Reproductive histories were collected using the same survey tool (Own Child Method) as was used with

women living in Nubri. Infant ages were typically given using the Nepali calendar rather than the Tibetan calendar, and exact birth dates were available for all infants in Kathmandu. However, to facilitate comparison to the Nubri sample, birthdates were rounded to the nearest month. Refusal rates in Kathmandu were higher, with 12% ( $n = 8$ ) of identified mothers declining to participate, typically citing “work” as the primary reason for refusal.

**Milk collection.** A single milk sample was collected from each mother using collection protocols described below (Miller et al., 2013; Ruel et al., 1997). Single samples collected mid-feeding were the most ethical sampling strategy for this population that has no history of bottle use, marginal nutritional status, and high rates of infectious disease (Miller et al., 2013).

Mothers who agreed were asked to delay feeding for 1 h prior to our scheduled visit. Between 6 and 10 am, mothers were visited in their homes, as this is the sampling window. To ensure compliance, interviews were typically conducted prior to the milk collection; so we could validate that a minimum of 1 h had elapsed since the prior feeding. Once the infant indicated hunger, the infant was weighed, and then allowed to nurse on one side for 2 min. After 2 min, the infant was removed from the breast. Mothers then hand expressed an 8–10 mL sample of milk into a sterile polypropylene container. After expression, the infant resumed nursing until satiated by that breast, at which point the infant was reweighed. This weigh–test–weigh methodology may underestimate the usual volume transferred, as this may have disrupted normal breastfeeding behaviors. Milk volume was only available on 93.9% ( $n = 77/82$ ) of the sample owing to issues with measurement (infant urination, toddler tantrum), or the challenges unique to the rural research site (e.g., yaks entering a research participant’s courtyard at an inopportune time).

Milk samples were transported on cold packs then aliquoted into 1.0 mL aliquots and frozen in liquid-nitrogen dewars (MVE XC Millennium 20) within 2 h of collection. Samples were transferred to dry ice for shipment back to the United States where they were stored at  $-80^{\circ}\text{C}$  prior to analysis.

**Anthropometric collection.** Anthropometrics were collected by three trained observers with high intraobserver reliability; interobserver variability was <10%. Height, weight, and mid-upper arm circumference were collected on all mothers. Skinfold thickness measurements were collected on mothers at the triceps, biceps, suprailiac, and subscapular skinfold sites using Lange calipers; and on infants at the triceps, biceps, suprailiac, subscapular, abdominal, thigh, and calf skinfolds. Infant recumbent length was measured using a portable infantometer. Infants were weighed before and after nursing to the nearest gram; because of the cold temperatures, infants were weighed with light clothing that was subtracted from final weight. All measurements were done in triplicate using established protocols (Frisancho, 1990; Rodríguez et al., 2005). Infant anthropometric Z-scores were calculated using the WHO standards from the Zanthro program for Stata 12.1, using month of birth and the approximate date function.



**Milk analysis.** Milk samples were analyzed at the Nutrition Lab at the Smithsonian National Zoological Park following standard protocols (Hood et al., 2009). Milk fat was measured using a micro-Rose Gottlieb procedure. Milk protein was determined by combusting total nitrogen on an Elmer–Perkins CHN analyzer and converting total nitrogen to protein (Power et al., 2008). Total sugars were measured using a micro phenol-sulfuric acid (DuBois) technique method (DuBois et al., 1951). All samples except sugar were run in duplicate; sugar samples were run in triplicate. Macronutrients were measured as percentage of weight then converted to grams per 100 mL. Milk energy was calculated using the standard formula for human milk:  $9.25$  (fat in grams) +  $5.65$  (protein in grams) +  $3.95$  (total sugar in grams) = milk energy (kcal/mL) (Garza and Butte, 1986); it should be noted that this is a per unit measurement of energy (milk energy density) not total milk energy which would be sensitive to both volume and energy density. Analyzing total sugar, rather than just lactose, results in slightly elevated milk sugar values compared to populations where only milk lactose was measured.

**Selection of comparative analyses in other populations.** The sampled populations included in Table 1 were selected from available published studies. Initial literature searches were conducted in PubMed and Google Scholar using the keywords “human milk composition”, “breast milk composition”, and “human milk macronutrient composition”. From the results, some 2,282 abstracts were available for review; the overwhelming majority did not focus on milk macronutrients and were excluded. Studies conducted before 1970 were excluded, as there is some evidence that methodological issues with collection, storage, and analysis may bias data collected before 1970. Studies not published in English were also excluded. To avoid overrepresentation of western, highly industrialized countries such as the United States or Australia, a single study was used as a representative measure for each population, with the most highly cited studies (for example, DARLING) used for that population. The remaining 69 abstracts were reviewed for both collection and analytical methods to determine inclusion.

For collection methodologies, mid feed, 24 h pooled samples, full mammary expression, and fore-hind-mid samples were eligible for inclusion while studies collecting only fore or hind milk were excluded. Studies combining milk from multiple mothers for a single measurement were also excluded. Analytically, only the following techniques were included: creamatocrit, Folch extraction, Rose-Gottlieb (including micro), and infrared analysis. Colorimetric measurements were excluded. Finally, studies must have included both a published sample mean and standard deviation for milk fat content. Studies that published modes, intraquartile ranges, or only included means in graphs were excluded as the true mean and standard deviation could not be known. The final group of eligible studies is included in Table 1. Only milk fat is listed, as the majority of available studies did not include data on milk sugar/lactose, or milk protein content, and limiting the sample to only papers with complete data on all macronutrients would have greatly reduced the sample size.

It is well established that collection practices can result in biased in milk macronutrient composition,

especially for fat and energy density (for review see Vilalpando and Del Prado, 1999; Miller et al., 2013). Milk fat changes over the course of a feeding, with milk fat, and consequently milk energy, increasing from the beginning to the end of the feeding (Daly et al., 1993; Kent et al., 2006). The magnitude of change in fat is driven by a number of factors, including storage capacity of the individual breast, time interval since last feeding, volume of current feeding, and other aspects of individual physiology (Daly et al., 1993). Samples collected at the beginning of a feeding will consequently have lower fat and less energy than samples collected at the end of the same feeding, or those from a full mammary expression. Mid-feeding sample collection attempts to overcome this, allowing the infant to nurse for 2–3 min prior to sample collection, and is generally considered to be a reasonable substitute for full mammary expression in populations where this would not be possible. Mid-feed samples may tend to underestimate milk fat compared to a complete expression. As we and others have discussed elsewhere (Miller et al., 2013), work with populations where bottles are not used—or medically fragile infants such as preterm infants—should preclude the use of full mammary expression, as it may either deprive the infant of the contents of the breast or, should the milk be bottle fed to the infant, carries ethical concerns associated with such use (Miller et al., 2013).

Time of day when samples were collected will also contribute to variation in total milk fat. There is good evidence that milk fat varies across the day, although the patterns of change vary by population. Among Guatemalan mothers, Ruel et al., (1997) reported that milk fat peaked in the mid-afternoon, from noon–2 pm, with the feeding between 6 and 10 am being most representative of a 24 h pooled sample ( $4.1 \pm 1.6\%$  milk fat in the sample versus  $4.2 \pm 0.9\%$  milk fat in the pooled sample studied by Ruel et al., 1997). Gambian mothers, by comparison, have fat peaks earlier in the day, between 9 and 11 am (Prentice et al., 1981b), with mid-day (1 pm samples) most reflective of 24 pooled averages. Australian mothers meanwhile have mid-day fat peaks and lower fat in early morning samples compared to day or evening samples (Khan et al., 2013). In all studies, there was considerable individual variation in addition to the patterns of diurnal changes in milk fat.

**Data analysis.** Analyses were conducted using Stata 12.1 (College Station, TX). Milk macronutrients, energy, and volume were log transformed prior to analysis. One-way ANOVA was used to test for differences by altitude two ways: first by average village altitude and second by using a three-level variable that stratified villages into groups based on altitude as follows: High rural (>10,000 ft), low rural (10,000–7,600 ft), low urban/Kathmandu (<5,000 ft), allowing for adjustment of secondary predictors (infant age, maternal parity, maternal adiposity). Multivariate regression analyses were then used to test for linear associations between milk composition and infant age, maternal age, three measures of maternal adiposity (BMI, triceps, and subscapular skinfold thicknesses—each modeled individually), parity, volume of milk transferred, and daily nursing frequency. Initial models also included average village altitude although this was not included in later models based on a lack of significance; maternal age was also dropped for lack of significance. Final models used maternal triceps

TABLE 1. Samples characteristics for the populations used for comparison. For each population, sample size, infant age, and collection and analytical technique are provided for comparison

Population	Sample size	Infant age	Collection technique	Analytical technique	Fat mean	Fat SD	Reference
Bangladeshi	58	6 months	24 h collection, pooled samples	Gravimetric	2.66	0.46	Brown et al., 1986
Pakistan	10	6 months	Full mammary expression	Rose-Gottlieb	2.72	0.13	Underwood et al., 1970
Ivory Coast	33	1–24 months	Morning, full mammary expression	Folch extraction	3.07	0.65	Lauber and Reinhold, 1979
Australian	17	4 months	fore/hind each feed, 24 h average	Colorometric	3.74	0.66	Mitoulas et al., 2002
America	92	0–12 months	Full mammary expression with 10 mL subsample collected across 24 h	Folch extraction	3.77	0.96	Nommsen et al., 1991
Gambia	120	6 months	Small samples, manual	Creamatocrit	3.93	1.65	Prentice et al., 1981
Philippines	124	<2 years	Manual expression after 2 min, 10 am	Micro Rose-Gottlieb	4.06	1.68	Quinn et al., 2013
Guatemala	52	1–4 months	Full mammary expression; 24 h pooled	Triglyceride	4.35	0.9	Ruel et al., 1997
Egyptian	24	<1 year	Manual expression of 10 fore/10 hind mixed	Gerber	4.43	0.89	Hanafy et al., 1972
Singapore	50	4 months	Full mammary expression	MIRIS	4.65	2.1	Thakker et al., 2013
Tibetan	83	7 days–3 years	Manual expression after 2 min, 10 am	Micro Rose-Gottlieb	5.24	2	This study

skinfold thicknesses as the measure of maternal adiposity. All secondary predictors were determined based on published reports in the literature (Butte and King, 2005; Prentice et al., 1994; Villalpando and del Prado, 1999). Bayesian information criterion (BIC) was used to test for differences in regression model, with the final model for milk fat showing a decline in BIC of 6.36 from the initial model and the energy model showing a decline of 2.21. Regression diagnostic techniques were also used on all models. As milk energy measurements are not independent of macronutrient measurements as macronutrients were used to calculate milk energy, regression models for milk energy should not be considered independent of regression models for other milk macronutrients, especially milk fat.

To compare the mean milk fat for this study with other populations, we used a Welch–Satterthaus *t*-test. As comparable, low-altitude populations differed in sample size and methodology, more conventional methods were not suitable to test for significant differences in milk composition between available sample populations, even after populations had been restricted by methodology. The Welch–Satterthaus test uses means and standard deviations were calculated from each published study weighted for sample size. The group mean, calculated from the available populations, was then compared to the sample mean for the combined sample studied here.

**Human subjects.** Human subjects' approval for the research was obtained from Washington University in St. Louis and the Nepal Health Research Council prior to the start of research. Upon entering each village, we made contact with local leaders, the local women's association, and village health workers. In community-based meetings, the project goals and protocols were explained

to the mothers prior to any recruitment. In Kathmandu, a similar practice was followed in each Tibetan community with Quinn and a field assistant making contact with community leaders.

## RESULTS

### Descriptive characteristics

The descriptive characteristics (mean, standard deviation) of the sample, stratified by altitude group (high rural, low rural, low urban), are shown in Table 2. There were no significant differences between mothers in the three groups for infant age, maternal BMI, maternal age, nursing frequency, or milk volume. However, mothers in the low urban group, living in Kathmandu, had lower parities than mothers in Nubri, reflecting reduced birth rates among urban Tibetans. Milk-specific characteristics for the groups are shown in Table 3.

### Lack of association with altitude

We found no association between altitude and measures of milk macronutrients, energy, or volume after adjustment for infant age (Table 4). Mothers living in high-altitude villages (>10,000 ft) did not produce milk that was statistically different in composition from mothers living at lower altitude villages (<10,000 ft) or in Kathmandu after adjustment for infant age, maternal adiposity, and maternal parity (Table 4). However, milk sugar was significantly associated with altitude of residence when no other predictors were included. The inclusion of maternal body composition (BMI or triceps skinfold thickness), infant age, or maternal parity in the model eliminated this association (Table 4). Most likely, as mothers living at higher altitudes tended to be

TABLE 2. Sample characteristics of mothers from Nubri and Kathmandu

Variable	All	High rural	Low rural	Low urban
N	82	26	15	41
Maternal height (cm)	153.0 (5.8)	154.3 (5.8)	155.0 (3.8)	151.4 (5.9)
Maternal weight (kg)	53.8 (7.6)	52.6 (6.6)	50.8 (6.6)	55.6 (8.2)
Maternal BMI (kg/m <sup>2</sup> )	22.6 (3.3)	21.7 (2.8)	20.7 (2.0)	23.9 (3.4)
Maternal triceps (mm)	14.6 (6.0)	11.9 (4.7)	10.0 (3.8)	17.9 (5.5)
Maternal age (years)	29.4 (7.2)	28.6 (7.1)	30.9 (8.5)	29.4 (6.9)
Parity	2.2 (1.4)	2.8 (1.4)	3.0 (1.5)	1.4 (1.0)
Infant age (months)	11.14 (8.11)	10.3 (6.6)	10.4 (9.1)	10.77 (7.94)
Weight (grams)	8080 (2159)	7577 (1837)	7416 (1297)	8656 (2474)
Length (cm)	70.2 (9.3)	67.6 (8.4)	68.3 (7.1)	72.6 (10.2)
BMI (kg/m <sup>2</sup> )	16.2 (1.9)	16.4 (1.7)	16.0 (1.9)	16.2 (2.3)
Infant sex (male)	48.7	47.8	66.7	46.3

TABLE 3. Descriptive characteristics of milk macronutrient and energy content

	ALL <i>n</i> = 82		Nubri, high <i>n</i> = 26		Nubri, low <i>n</i> = 15		Kathmandu <i>n</i> = 41	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fat (g/100 mL)	5.24	2.01	4.57	1.51	4.75	1.62	5.72	2.29
Sugar (g/100 mL)	7.37	0.49	7.25	0.35	7.34	0.32	7.19	0.55
Protein (g/100 mL)	1.26	0.35	1.18	0.19	1.15	0.12	1.33	0.47
Energy (kcal/100 mL)	81.38	17.36	77.23	13.49	78.98	14.7	85.15	19.93
Frequency ( <i>n</i> /24 h)	8.7	2.2	8.6	1.6	9.7	1.1	8.4	2.7
Volume (mL)	44.7	36.8	46.6	47.2	45.8	22.3	38.2	33.9

heavier (Table 3), the altitude effect was confounding with maternal characteristics.

### Comparisons with other studies populations

Welsh-Satterthausen *t*-tests were used to calculate a single summary mean for prior studies. Mean milk fat for the combined studies was  $3.90 \pm 1.23$  g/100 mL, compared to the full sample mean of  $5.24 \pm 2.00$  g/100 mL from this study. Based on calculations for Welsh-Satterthausen, these sample means are significantly different ( $p > 0.01$ ) for milk fat (Table 1).

### Predictors of milk macronutrient composition in this sample

There were no associations between milk volume and milk macronutrient or energy content in this sample. Mean nursing frequency per day was  $8.6 \pm 1.6$  bouts for high-altitude rural mothers,  $9.7 \pm 1.1$  bouts for low-altitude rural mothers, and  $8.4 \pm 2.7$  bouts for urban mothers; differences were not significant. Mean volume transferred per measured feeding was  $46.6 \pm 47.2$  mL for high-altitude rural mothers,  $45.8 \pm 22.3$  mL for low-altitude rural mothers, and  $38.2 \pm 33.9$  mL for low-altitude mothers using weigh-test-weigh. Mean infant ages for all groups were not significantly different.

In this sample, mean triceps skinfold thickness for mothers was  $14.6 \pm 6.0$  mm, approximately the 15<sup>th</sup> percentile of skinfold thicknesses compared to reference values from the United States (McDowell et al., 2008). Maternal triceps skinfold thickness were significantly higher in mothers living in Kathmandu compared to the two samples from Nubri ( $17.9 \pm 5.5$  mm Kathmandu vs  $11.9 \pm 4.7$  mm high rural,  $10.0 \pm 3.8$  low rural;  $p < 0.000$ ).

Maternal BMI had a nonsignificant, positive association with milk fat ( $p < 0.089$ ) and an inverse association

with milk sugar ( $p < 0.051$ ). Maternal triceps skinfold thickness was significantly associated with milk fat ( $p < 0.02$ ) and sugar ( $p < 0.013$ ). Each 1 mm increase in triceps skinfold thickness predicted a 0.09 g/100 mL (0.03 g/100 mL) increase in milk fat after adjustment for infant age and maternal parity, and a corresponding decrease of  $-0.08$  g/100 mL (0.04 g/100 mL) for milk sugar, after adjustment (Table 4).

Milk fat was significantly and positively associated with infant age (Table 4). Each 1 month increase in infant age predicted a 0.10 g/100 mL (0.02 g/100 mL) increase in milk fat after adjustment for infant age, parity, and maternal triceps skinfold thickness (Table 4). Infant age was not a significant predictor of milk sugar; however, milk sugar was positively associated with maternal parity. Higher maternal parity predicted decreased milk fat, although the association did not reach significance ( $B = -0.229$ ,  $p < 0.079$ ).

Milk energy density (kcal/100 mL) was significantly associated with both maternal triceps skinfold thickness and infant age, but not parity or BMI. As milk fat contributes the greatest percentage of calories to milk energy, it is not surprising that the same predictors would be significant for both milk fat and milk energy density. There was no association between any predictor and milk protein content (Table 4).

There were no associations between maternal dietary intakes and milk composition or volume in this sample, based on one 24 h maternal dietary recall; dietary recalls were likely biased reflecting local eating patterns, such as continually refilling tea cups.

### No association between milk macronutrients and infant anthropometrics

Milk macronutrient and energy content were not significantly associated with infant weight or length *z*-score

TABLE 4. Regression models testing for an association between primary and secondary predictors and milk composition

Variable	Milk fat			Milk sugar			Milk protein			Milk energy		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
Altitude (ft/sea level)	-0.149 (0.085)	-0.046 (0.095)		0.057 (0.021)**	0.032 (0.024)		-0.015 (0.016)	-0.001 (0.018)		-1.219 (0.768)	-0.297 (0.852)	
Infant age	0.086 (0.025)**	0.100 (0.025)**	0.103 (0.024)**	-0.007 (0.006)	-0.010 (0.006)	-0.011 (0.006)	0.002 (0.005)	0.004 (0.005)	0.003 (0.005)		0.893 (0.223)**	0.920 (0.216)**
Maternal BMI		0.113 (0.063)			-0.022 (0.016)			0.013 (0.012)			1.022 (0.571)	
Maternal parity		-0.260 (0.152)	-0.229 (0.137)		0.073 (0.038)	-0.017 (0.005)*		-0.039 (0.030)	-0.039 (0.028)		-2.303 (1.368)	-1.984 (1.241)
Maternal triceps			0.095 (0.033)**			-0.083 (0.036)**			0.002 (0.006)			0.812 (0.294)**
Constant	4.562 (0.425)	2.114 (1.599)	3.038 (0.695)	7.014 (0.105)	7.455 (0.400)	7.296 (0.181)	1.245 (0.080)	0.973 (0.310)	1.244 (0.140)	76.581 (3.826)	54.367 (14.397)	63.754 (6.287)
R2	0.141	0.178	0.218	0.073	0.107	0.104	0.010	0.000	0.006	0.134	0.171	0.215

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

after adjustment for age, sex, and parity. Neither nursing frequency nor milk volume transferred were significant predictors of infant size.

DISCUSSION

Adaptation to high altitude

Contrary to our initial hypothesis that there would be a dose-dependent association between altitude and milk macronutrient composition and volume in this sample, no such association was identified. However, mean milk fat for this sample was higher than mean milk fat for a subset of previously studied populations (Table 1). Neither protein nor sugar means were outside of the range of prior populations (data not shown). While individual means were available for the populations included in Table 1, larger populations tended to have more variation, and most samples had overlapping distributions with this study. At present, no comparative data for high-altitude adapted human populations exist, but there is some evidence that yaks living at high altitude produce milk with higher fat than yaks of the same breed living at lower altitude (Barsila et al., 2014).

Alternatively, if the mean milk fat measured in this sample is representative of a general tendency toward increased milk fat and energy for high-altitude populations, these changes may be offset by a reduction in milk volume. The Tibetan infants in this study may receive high-fat/high-energy milk, but this is coupled with a reduction in volume, making their energy intakes—and the energy costs to mothers—no different from those of low-altitude populations. It has long been hypothesized, but not empirically tested, that the metabolic costs of infancy may be elevated for high-altitude infants. However, BMR or its more frequently used infant equivalent, sleeping metabolic rate (SMR), has never been measured in a Tibetan infants. A higher SMR is inferred based on increased metabolic rates in adults and older children. If true, increased metabolic rate should require either increased energy intake during infancy or trade-offs from other systems, such as decreased growth velocity, something also observed in this sample. Greater energy transfer, if present, may be a mechanism for meeting increased metabolic needs during infancy, part of what Vitzthum (2001) has dubbed “the home field advantage” of high-altitude adapted reproduction, but would likely increase maternal costs. Further research, using longitudinal study designs and high-intensity test weighing, will be necessary to elucidate metabolic associations between maternal condition, milk composition, and infant metabolism.

Higher milk fat and energy content should predict increased energetic costs for mothers who must bear the increased metabolic costs of milk synthesis (Prentice and Prentice, 1988). Tibetan mothers also report desired breastfeeding durations of 2–3 years, and have relatively high fertility, showing no evidence for a reduction in fertility outside of what would be expected given lactation lengths (Vitzthum and Wiley, 2003). The application of an indirect means to estimate fertility (The Own Children Method) in five of the study villages revealed a total fertility rate of 5.9 births per woman during the 1998–2012 time span. This is consistent with fertility estimates from the early 1990s that ranged by village from 5.6 to 7.0 births per woman (Childs, 2004a).



### Genetic legacies of adaptation to high altitude

There were no differences in milk composition between urban/rural or high-/low-altitude living Tibetan mothers. All study groups had a tendency toward higher milk fat, and consequently milk energy. One possible interpretation of these findings is that this may be part of more generalized patterns of adaptation to high altitude. In this scenario, we hypothesize that increased milk fat and energy may be a means of meeting increased infant metabolic demands theoretically present during high-altitude infancy. This may be part of the mechanism contributing to increased survival rates in Tibetan infants compared to infants from populations with more recent migration to high altitude, such as Han Chinese. A single study of milk composition among low-altitude-living Han Chinese reported a mean milk fat of 4.3 g/100 mL (Yang et al., 2014). Prior studies have shown increased mortality of Han infants at high altitude compared to Tibetan infants despite greater household wealth of the Han; differences in daily energetic intake may contribute to increased mortality (Moore et al., 2006). Although metabolic studies have not been conducted on infants living at high altitude, the altered growth patterns seen in these offspring, both pre- and postnatally, have been interpreted as adaptations to high altitude (Beall, 1981; Dang et al., 2008; Moore et al., 2001; Vitzthum, 2001; Weitz et al., 2004; Wiley, 1994).

A growing body of evidence suggests that the hypoxia inducible factor system (HIF) may have been a major target of selection among high-altitude adapted populations (Beall et al., 2010; Petousi and Robbins, 2013; Simonson et al., 2010). The hypoxia-inducible factor system is a group of oxygen-sensitive transcription factors that play important roles in maintaining oxygen homeostasis across numerous different tissues. They are essential to all mammalian life, and have functional roles in the development, maturation, and maintenance of numerous tissues. In environments where chronic hypoxia may present ongoing physiological challenges, there is evidence for selection of HIF-related genes. Tibetans, for example, may have a hypo-responsive HIF transcriptional system, as measured using HIF-2 $\alpha$  from peripheral blood leukocytes (Petousi et al., 2014). It has been demonstrated in knock-out mice models that another of the hypoxia-inducible factors, HIF-1 $\alpha$ , is important for secretory differentiation and activation of the mammary epithelium. HIF-1 $\alpha$  knock-out mice have highly viscous milk, suggestive of increased fat content (Seagroves et al., 2003). Further research, incorporating genetic data and possibly candidate genes involved in both milk synthesis and high-altitude adaptation, will be necessary to test these hypotheses.

### Maternal triceps skinfold thickness, but not diet predicts milk fat

Prior studies have reported a positive association between maternal BMI and milk fat and/or energy (Butte et al., 1984; Prentice et al., 1994; Villalpando and del Prado, 1999), although not all studies have reported this association (Butte and Calloway, 1981; Quinn et al., 2012). Here, we found that maternal triceps skinfold thickness was a better predictor of milk fat, sugar, and energy than maternal BMI. Similar associations between triceps skinfold thicknesses and milk macronutrients

have been described in other populations (Brown et al., 1986; Nommsen et al., 1991; Villalpando et al., 1992).

However, the utility of maternal triceps skinfold thicknesses for cross-population comparisons is much more limited. The strength and magnitude of the associations between skinfold thicknesses and milk fat vary across populations, such that it is impossible to use maternal triceps skinfold thickness as a substitute for milk fat. For example, Brown et al., (1986), in a sample of 60 Bangladeshi mothers, reported higher milk fat in mothers with triceps skinfold thicknesses >11 mm when compared to mothers with triceps skinfold thicknesses below 8 mm. By comparison, in a sample of 58 mothers nursing 3-month-old infants from the United States, mean triceps skinfold was much higher than that reported for Bangladeshi mothers while mean milk fat was lower (Nommsen et al., 1991). Longitudinal studies from Bangladesh and The Gambia have shown that declining skinfold thickness predicts decreased milk fat within these groups (Brown et al., 1986; Prentice et al., 1981). Here, we report relatively low maternal triceps skinfold thickness (14.6  $\pm$  6.0 mm) but high milk fat (5.24  $\pm$  2.01 g/100 mL).

There were no associations between maternal dietary intakes and milk macronutrient composition in this sample. While this is similar to findings reported by prior studies, the possibility of sample bias cannot be ruled out. Maternal dietary intakes were predominantly centered on tsampa (roasted barley flour mixed with small amounts of butter tea), butter tea, potatoes, rice, and lentils. The primary source of dietary fat was butter, made from the milk of *Bos grunniens* or *Bos primigenius taurus*. This butter is sometimes mixed into tsampa but more commonly consumed as a beverage after being churned with tea and salt. Although women reported drinking only two cups of butter tea per day, observation provides evidence that actual consumption is much higher. Therefore, the reported two cups a day actually represents two tea-drinking sessions per day, with total consumption well exceeding two cups. We think available dietary data actually underestimate maternal consumption, especially for dietary fat. However, as prior studies and several reviews have found weak to no association between daily energy intakes and milk macronutrient composition (Villalpando and del Prado, 1999), these difficulties with dietary recalls are unlikely to bias study results.

Although hypoxia is often viewed as one of the primary stressors for high-altitude adapted populations, seasonal and chronic food shortages from a truncated growing season are also sources of physiological stress (Baker, 1978). The association between milk fat and energy and maternal triceps skinfold thickness reported here may be an example of these well-established population trends, with under-nourished populations potentially using maternal physiological capital as a buffering mechanism for energy shortfalls (Villalpando and del Prado, 1999).

### Life history trade-offs in adaptation to high altitude

Humans have a wide and highly varied geographical range. While behavioral complexity has certainly facilitated human capacity to colonize novel environments, human biological plasticity has likely played an important role (Wells and Stock, 2007). Wells (2010) has



suggested that the increased adiposity of humans may facilitate their capacity to colonize new environments and to support reproduction in variable or low quality environments. One of the critical periods would be during lactation, and may explain why humans, compared to other primates, can provide some degree of nutritional buffering to nursing infants. However, as prior research into metabolic function among high-altitude living Tibetans has found, adults have increased basal metabolic rates, with female BMR on average 8% higher than comparable women living at sea level (Beall et al., 1996). It is unknown, however, if BMR remains elevated during lactation, as BMR is generally suppressed in lactating women at lower altitude (Butte et al., 1999; Butte and King, 2005). It may be that the constraints of high altitude limit maternal physiological capacity to down-regulate basal metabolic rates. While human evidence is unavailable, murine models have shown that chronic cold stress predicts a decrease in milk volume (Krol et al., 2011), thought to reflect an inability to down-regulate maternal thermoregulatory costs.

Estimates for the cost of milk synthesis in humans give a measure of 323–336 kJ/100 mL about 77.19–80.3 kcal/100 mL maternal energy per 100 mL of breast milk synthesized, assuming protein concentrations of 1.2 g, lactose content of 7.5 g, and fat concentrations of 4.0 g and no additional costs of transport or synthesis of precursors (Prentice and Prentice, 1988); variation in the synthesis of individual macronutrients will alter costs for mothers—for example, producing the mean milk fat for the full sample, 5.2 g, may or may not increase the costs of milk synthesis for mothers depending on dietary fat intake. Birth intervals for women at high altitude show no evidence for a reduction in fertility outside of what would be expected given lactation lengths (Vitzthum and Wiley, 2003).

Numerous pregnancies and long lactations will be energetically costly to mothers, and may reduce bodily resources to sustain lactation. These costs may contribute to the borderline association between maternal parity and milk fat and energy, as well as the positive association between maternal triceps skinfold thickness and milk fat, as mothers with higher body fat should have improved resources to support reproduction.

### Limitations

This study is limited in that milk composition was only measured in a single sample, and as such, all data are cross-sectional. Moreover, the sampling methodology may have biased the fat and energy content of the milk, as discussed above. While volume and feeding frequency were within the range reported by other populations, we cannot rule out the possibility that mothers may compensate for high-fat milk by reducing overall milk volume, as 24 hour milk intakes were unavailable. However, the unusually high fat content of milk, paired with volumes, milk sugar, and protein contents fall within the range reported for other populations.

### CONCLUSIONS

The plasticity of the human phenotype and the capacity of genetic adaptation over relatively short time periods to cope with high altitudes are well established (Beall, 2006; Moore et al., 2006; Simonson et al., 2010), with a growing body of evidence demonstrating the interaction between environment, genotype, and the pro-

duction of high-altitude adapted phenotypes (Beall et al., 2012; Petousi, 2013). Human biological variation extends to milk composition, which is in itself a phenotype, and has likely been under selection. While we did not identify a dose association between milk fat and the current altitude at which the mother lived, we did find a comparatively high mean milk fat for the Tibetan mothers in this sample.

This research illustrates the potential for human milk to have contributed to the evolutionary success of humans at high altitude. Buffering of milk macronutrients and energy may be part of the human adaptive complex, facilitating colonialization of novel environments. Milk is itself ultimately a phenotype, one that is produced by an existing phenotype (the mother) and will influence the subsequent development of offspring phenotype (Kuzawa and Quinn, 2009). This should be important in the construction of ecogeophenotypes in children that may promote survival (Cowgill et al., 2012), especially in novel environments where short-term phenotypic plasticity may be important (Wells, 2012).

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