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RESEARCH PAPER

Ecological pressures and milk metabolic hormones of ethnic Tibetans living at different altitudes

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ABSTRACT

Background: Very little is known about how milk hormones, shown to influence growth during infancy, may contribute to patterns of altered growth in high altitude living infants.

Aim: This study investigated the association between maternal BMI, the metabolic hormones adiponectin and leptin in human milk and infant weight for age z-scores (WAZ) in Tibetans.

Subjects and methods: A sample of 116 mothers and infants (aged 0–36 months) were recruited from two locations: the Nubri Valley, Nepal (rural; altitude = 2400–3900 m) and Kathmandu, Nepal (urban, 1400 m). Milk samples, anthropometrics, biological data and environmental information were collected on mothers and infants. Milk was analysed for leptin and adiponectin.

Results: Maternal BMI was significantly associated with milk leptin content, but not adiponectin in either group. In the rural high altitude sample, child WAZ declined with age, but no such decline was seen in the urban sample.

Conclusions: Milk leptin and adiponectin were not associated with infant growth in the rural Nubri sample, but were both inversely associated with infant WAZ in the Kathmandu sample. It appears that, in ecologically stressful environments, associations between milk hormones and growth during infancy may not be detectable in cross-sectional studies.

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Introduction

The physiological challenges of living at high altitude are well described (Beall, 2007; Moore et al., 1998, 2006). Considerable research has investigated the multitude of ways in which populations have adapted to these challenges, identifying considerable variation in both biological and behavioural strategies across high altitude adapted populations (Moore et al., 1998). There is good evidence for genetic adaptation to high altitude (Beall, 2007), again with multiple genetic strategies underlying these adaptations (Beall et al., 2004; Moore et al., 2006; Xu et al., 2014). Selective pressures from high altitude will occur across the lifespan of individuals, with primary pressures differing over the life course (Moore et al., 1998; Beall, 2013). For example, infants and children may be more sensitive to cold stress than adults. Intensive selection on phenotypes will occur throughout life, but, as we and others have argued, such ecologically driven selection may be greatest during infancy and early life (Kuzawa & Quinn, 2009; Wells, 2014). Developmental plasticity, allowing for changes to the phenotype in response to ecological pressures, may be an important mechanism for promoting survival. It was recently shown that immature phenotypes are more likely to display classical patterns of environmental adaptation than adult phenotypes (Cowgill et al., 2012; Wells, 2012). Wells and Stock (2007) have argued that this early developmental

plasticity may be an important hallmark of human biology and the foundation for the human capacity to occupy novel and often stressful environments.

Multiple authors suggest that exposure to certain hormones during development may be instrumental in organising specific phenotypes (Palou et al., 2011; Picó et al., 2007; Singhal & Lanigan, 2007; Weyermann et al., 2007). One likely candidate for providing these early organisational signals is human milk (Bartol et al., 2013; Kuzawa & Quinn, 2009; Savino et al., 2009, 2013). Many of the hormones with known organisational effects on immature physiology are found in milk. It is likely that these milk-borne hormones should promote developmental trajectories appropriate for the lived ecology while balancing infant metabolic demands, maternal reproductive costs and ecological risk factors—both pathogenic and those associated with unusual or extreme environments (Quinn et al. 2015).

Until recently, human milk has not been investigated in populations living in unusually ecologically stressful conditions, such as high altitude. Previously, we have demonstrated that milk macronutrients and consequently dependent milk energy are buffered from ecological pressures associated with high altitude (Quinn et al., 2016). Similar findings for milk macronutrients have been demonstrated for most human populations living under nutritional or pathogenic stress (Brown et al., 1986; Jelliffe & Jelliffe, 1978; Prentice, 1994; Prentice, 1995; Villalpando & del Prado, 1999), although there

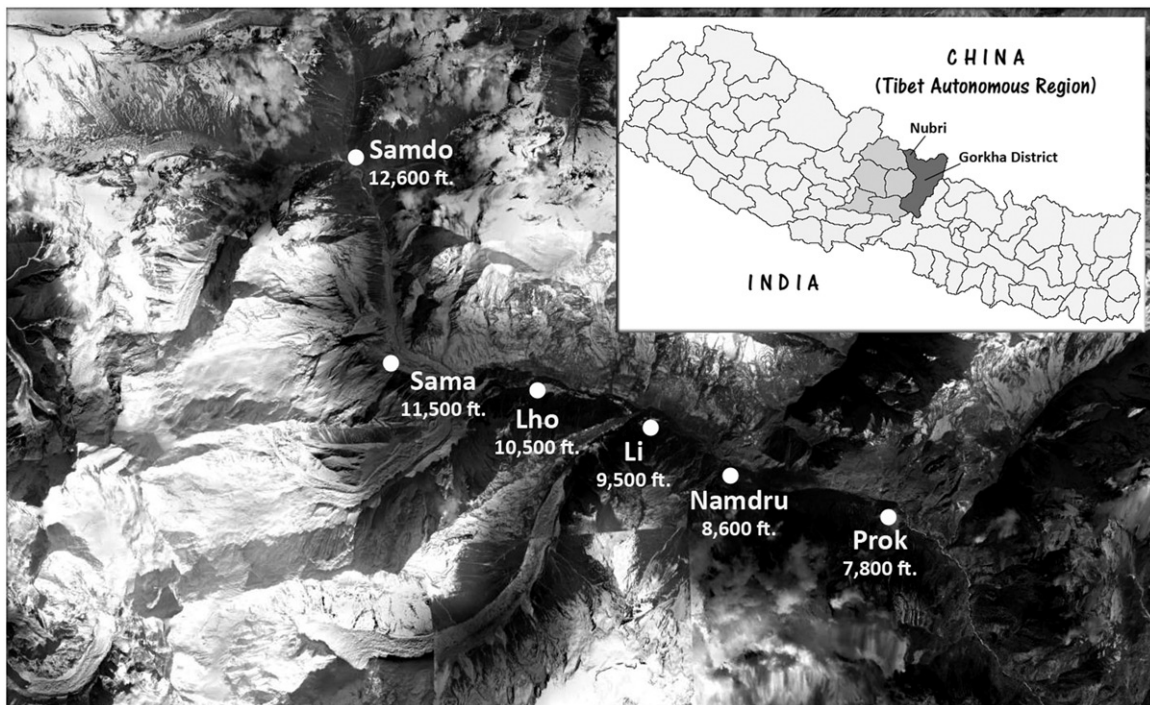


Figure 1. Map of the six primary villages of the Nubri Valley, Nepal. Image: Childs.

is some evidence for within-group associations between maternal BMI, parity and milk fat and milk lactose.

However, it is the hormones in milk, not the macronutrients, that have been hypothesised to be primary factors involved in lactocrine programming (Bagnell et al., 2009; Bartol et al., 2013). These hormones may provide organisational and developmental signals that promote ecologically adapted phenotypes. Here, we report on two hormones from mothers' milk thought to have important functions for short- and long-term developmental programming: the adipokines leptin and adiponectin studied in a sample of high altitude adapted ethnic Tibetans.

Both longitudinal and cross-sectional studies have reported significant associations between milk leptin and adiponectin and infant weight, either using change in weight velocity or weight for age z-scores (WAZ). Milk leptin is inversely associated with infant WAZ, as well as weight and BMI velocities across the first year of life (Brunner et al. 2015; Dundar et al., 2005; Doneray et al., 2009; Fields & Demerath, 2012; Miralles et al., 2006; Schuster et al., 2011; Quinn et al. 2015; Uysal et al., 2002). Milk adiponectin is also inversely associated with infant WAZ and weight velocity over the first year of life (Brunner et al. 2015; Weyermann et al., 2007; Woo et al., 2009). Higher milk adiponectin has been hypothesised to predict slower weight gain during the first year of life, but faster growth during the second year of life (Woo et al., 2012); at least in well-nourished populations.

Prior analyses have been primarily conducted on well-nourished populations with optimal growth velocity during the first year of life. It is less clear if these associations between milk metabolic hormones and infant weight gain may persist in populations with less optimal growth or how ecological stressors may influence these associations. We

investigated these associations among ethnic Tibetans, working with two cross-sectional samples of mothers and infants—one sample living at high altitude in six rural villages from urban Kathmandu, representing a low altitude sample. Three primary research questions were investigated: (1) are there associations between maternal adiposity and milk leptin and/or adiponectin; (2) are there associations between altitude of residence and milk hormones; and (3) are there associations between milk hormones and infant size for age?

Methods

Mothers and their infants were recruited from two project sites stratified by altitude: the Nubri Valley, Nepal and Kathmandu, Nepal.

Population—Nubri

Located in the Gorkha district of Nepal (Figure 1), Nubri is an ethnically Tibetan enclave, populated by roughly 3000 inhabitants. The valley is organised into small agricultural villages ranging in altitude from 2090–3830 metres. The people of Nubri are primarily descendants of migrants from the northern Tibetan Plateau with some migration from the lower valley; occupation and transborder migrations have been ongoing for the last 700 years (Childs, 2004). In Nubri, households primarily practice small-scale farming (barley, potatoes, maize) and animal husbandry (yak, yak-cow crossbreeds). Additional sources of income are more recent: some villages are involved in the trans-Himalayan trade of timber and medicinal plants, while others are part of the emerging mountaineering industry (Childs & Choedup, 2014). We recruited

mothers and infants from six of the larger villages: three located above 3000 metres and three located below 3000 metres; all were above 2000 metres.

Population—Kathmandu

The low altitude comparison group was recruited from Tibetan communities and refugee camps in Kathmandu, Nepal. All participants self-identified as Tibetan and were recruited through local mothers' groups, using snowball sampling. Mothers varied in the amount of time they had spent at low altitude—some had moved to Kathmandu as adults (either from Nepal or Tibet), while others had been born in Kathmandu. Data on duration of residence in Kathmandu was not collected. Approximately half the mothers in the Kathmandu sample were engaged in wage labour. Maternal education ranged from primary school to college.

Sample for analyses

A total of 136 women and their infants were recruited. Milk samples were obtained from 133 of these mothers. Final analyses were conducted on 116 milk samples—excluding women nursing infants greater than 36 months of age, mothers with limited milk volumes and mothers who were concurrently pregnant. The final sample consists of 50 women from Nubri and 66 women from Kathmandu; power calculations determined that 42–46 participants per group would be necessary to detect an effect size with $\alpha = 0.05$ with a power of 0.8 and 0.95, respectively.

Anthropometric measurements

Anthropometrics were collected by three trained observers with high intra-observer reliability; inter-observer reliability was less than 10%. Height, weight and mid-upper arm circumference were collected on all mothers using portable scales and stadiometers (Seca 213, Germany). Skinfold thickness measurements were collected on mothers at the triceps, biceps, suprailiac and subscapular skinfold sites using Lange calipers; skinfold measurements were collected on infants at the triceps, biceps, suprailiac, subscapular, abdominal, thigh and calf skinfolds. Infant recumbent length was measured using a portable infantometer (Seca 417, Germany). All measurements were done in triplicate using established anthropometric protocols (Frisancho, 1990; Rodríguez et al., 2005). Infant weight was measured to the nearest gram before and after nursing to measure volume transferred (Tanita BD-815U, Japan). Infants were weighed before and after nursing. The weight measurement prior to nursing was used as the measure for infant weight. Milk volume was calculated by dividing the weight change of the infant from before and after nursing by the specific gravity of milk, 1.036, and then added to the sample volume collected to calculate total milk volume transferred during the nursing session.

Infant anthropometric z-scores were calculated using the WHO Anthro program v3.2.2, using month of birth and the 'approximate date' function. Mothers were asked to provide

infant date of birth or, at a minimum, birth month and a year using the Tibetan calendar. For mothers in Kathmandu, exact birthdates were usually available, but to facilitate comparison with the less exact dates from Nubri, only the month and year were used.

Health data, reproductive histories and infant feeding

At each in-home visit, mothers were asked a series of questions about infant health over the past month. These included an illness recall, including symptoms and duration and any information on treatment. Maternal illness history over the past month was also collected, along with recall information for all other household members stratified into <6 years, 6–16 years and greater than 16 years of age. Mothers were also asked to recall infant feeding histories from birth, such as timing and use of non-human milks (dzo (female yak), cow, formula), use of colostrum, cross-nursing and the use of non-milk foods, such as pap.

Milk sampling

Milk samples were collected by hand expression between 6 am and 10 am, using protocols in widespread use (Miller et al., 2013; Ruel et al., 1997). Mothers were interviewed first in their homes to make sure the infant was not breastfed for a minimum of 1 hour prior to the study visit. When the infant was ready to nurse, the infant was weighed and then allowed to nurse for 2 minutes from either breast. After 2 minutes, the infant was removed from the breast and mothers manually expressed 8–10 mL of milk into a sterile polypropylene container (Medela 87061). The infant was then allowed to return to the breast and nurse until satiated, at which point the infant was re-weighed (Scanlon et al., 2002). Such minimal milk collection, utilising small volumes collected 2–5 minutes after the onset on nursing, are frequently used in low resource settings such as Nubri. As we have discussed elsewhere (Miller et al., 2013), although optimal collection techniques require full mammary expression, this is often not possible in low resource settings, especially in such settings where any form of mammary expression is unusual and bottles are rare. Under these field conditions, full mammary expression would have either deprived the child of nutrients, required introducing a bottle to a non-bottle using population or resulted in too large a sample volume to manage with the portable liquid nitrogen tanks. As such, we cannot collect full mammary expression and rely instead on the mid-feed sampling method as described in detail by Neville, (1984), albeit for the collection of lipids over hormones; similar validation studies for hormones have not been conducted.

Expressed milk samples were immediately placed on cooler packs and transported back to the portable lab at base camp where samples were aliquoted and frozen in liquid nitrogen dewars (MVE XC Millennium 20) within 2 hours of sample collection. At present, it is not thought that time of day contributes to variation in milk hormonal content (Bonnet et al., 2002; Bielicki et al., 2004; Casabiell et al., 1997),

although there is emerging evidence for a slight increase in milk leptin content from 17:00 to 24:00 hours, with a peak between 22:00 and 04:00, at least in Australian mothers (Cannon et al., 2015). While it appears that milk leptin content does increase slightly from the beginning to the end of a single feeding, the increase is not statistically significant (Cannon et al., 2015; Karatas et al., 2011). There is also no present evidence for associations between human milk leptin content and the interval between feedings or the length of a single feeding, with decreased milk leptin content only seen when large volumes of milk (>105 grams) were transferred (Cannon et al., 2015). No comparable human data exist for milk adiponectin. However, in a recent study using rat dams expressed at 4-hour intervals, milk leptin was consistent across the day, while milk adiponectin showed some variation across the day for samples collected on days 5 and 10 of lactation, but not for samples from day 15 (Nozhenko et al., 2015). In the rat dams, milk adiponectin peaked at noon in the early samples, but showed little diurnal change in milk samples from day 15.

Milk samples were transferred to dry ice for international shipping from Kathmandu to the US.

Laboratory analyses

Milk samples were analysed for hormones at the Biomarkers and Milk Laboratory. All samples were thawed and skimmed prior to analysis, with assays run on the skimmed milk; samples were sonicated prior to analysis.

Milk leptin was analysed using a modified commercially available kit from R & D Systems (DY398); we and others have previously published this protocol (Miralles et al., 2006; Quinn et al. 2015). Undiluted milk samples were analysed, as initial tests identified low levels of leptin in milk samples. Briefly, polystyrene single use plates were coated in capture antibody diluted in PBS. Coated plates incubated overnight at 4°C. The following morning, plates were blocked with reagent diluent for 2 hours. Standards and samples were then added and the plate incubated at room temperature with shaking at 500 rpm for 2 hours. Detection antibody was then added following the manufacturer's protocols, with an additional 2 hours of incubation at room temperature with shaking at 500 rpm. Streptavidin-horseradish peroxidase, followed by colourimetric substrate (R & D Systems DY999) was then used, following the manufacturer's protocols. Plates were read at 450 nm with correction to 540 on a Biotek Elx800. Intra-assay variation was 17.8%, within acceptable variation for hormonal assays.

Milk adiponectin was also analysed using a modified commercially available kit from R & D Systems (DY1065) using protocols we and others have previously published (Anderson et al., 2015; Bernstein & Dominy, 2013). Skimmed milk samples were diluted 1:5 in reagent diluent prior to assay. Plates were prepared according to the manufacturer's protocols and were incubated overnight at room temperature. All incubations occurred without shaking. Plates were read at 450 nm with correction to 540 nm on a Biotek

Elx800. Intra-assay variation was 14.4%, within acceptable ranges for variation.

Statistical analyses

All statistical analyses were run using Stata 12.0IC. Means and standard deviations were calculated from the full sample, then independently for each location (Nubri, Kathmandu). *T*-tests were used to test for differences between the two samples when data were normally distributed; non-normal data were log-transformed prior to the analyses if necessary.

Using *t*-tests, we identified that the Nubri and Kathmandu samples were statistically different and separate models were used for all further analyses. Regression diagnostics were used throughout. All regression models were tested using Akaike information criterion (AIC) for model testing; information is included in the regression tables.

To investigate hypothesis 1, Pearson's correlations were used to test for correlations between maternal BMI and milk hormones, using untransformed data as Pearson's correlation does not assume normality. Multivariate linear regression was then used to test for a linear relationship between maternal BMI and milk hormones, allowing for adjustment of infant age, nursing frequency and transferred milk volume. The regression models for the Nubri sample were then run with altitude as an additional predictor, as a test of hypothesis 2. To ease interpretations, altitude was run in thousand foot increments instead of one foot increments—7.8 thousand feet instead of 7800 feet.

For the final hypothesis, multivariate linear regression models were then used to test for associations between milk leptin and adiponectin and infant WAZ in the two groups. This was done using three models: leptin as a predictor of WAZ, adiponectin as a predictor of WAZ and a model including both leptin and adiponectin as predictors of WAZ. All models were run before and after adjustment for infant age, transferred milk volume, infant sex, birth order, nursing frequency and, in the Nubri sub-set, altitude of residence.

Results

Milk adiponectin and leptin levels were low in this sample compared to other populations, with mean leptin at 0.27 ± 0.25 ng/mL and mean adiponectin at 4.1 ± 2.13 ng/mL. There were significant differences in most characteristics between the two samples, with Kathmandu mothers being heavier and of lower parity than Nubri mothers (Table 1). Leptin levels were significantly higher in the milk of mothers from Kathmandu (0.35 ± 0.33 ng/mL vs 0.20 ± 0.13 ng/mL); this likely reflects the significantly greater BMI of mothers from Kathmandu compared to mothers from Nubri (Table 1). Mothers living in Nubri had significantly more adiponectin in their milk compared to mothers living in Kathmandu (4.39 ± 2.25 ng/mL vs 3.60 ± 1.89 ng/mL; $p < 0.024$). Maternal BMI was significantly associated with milk leptin in both groups (Table 2; Figure 2). Although models were adjusted for infant age, nursing frequency and milk volume transferred, the inclusions of these variables did not improve the models

Table 1. Descriptive characteristics of mothers and infants from Nubri and Kathmandu, Nepal.

Variable	Nubri Valley		Kathmandu		<i>p</i> -value diff
	<i>(n</i> = 64)		<i>(n</i> = 49)		
	Mean	SD	Mean	SD	
Mother age (years)	29.47	7.35	29.59	6.22	0.491
Birth order	2.88	1.53	1.55	1.14	0.000
Maternal body fat (%)	22.76	4.36	27.43	4.37	0.000
Maternal height (cm)	154.16	6.15	152.67	5.43	0.021
Maternal weight (kg)	52.79	6.20	56.39	8.25	0.001
Infant age (months)	11.02	7.66	11.70	8.44	0.211
Infant weight (g)	7775.39	1608.31	8968.84	2761.54	0.000
Infant head circumference (cm)	43.17	5.16	44.16	4.51	0.145
Nursing frequency	8.80	1.98	8.14	3.08	0.006
Infant length (cm)	68.47	7.79	73.33	10.86	0.000
Infant male (%)	0.53	0.50	0.51	0.51	0.681
Age infant supplemented (days)	18.16	28.48	80.76	59.54	0.000
Milk volume (mL)	41.34	35.53	36.41	31.57	0.251
Milk adiponectin (pg/mL)	4470.84	2239.43	3732.52	1926.73	0.020
Milk leptin (ng/mL)	203.60	126.85	354.03	339.16	0.004

and the unadjusted model remained the best fit model. For each one unit increase in maternal BMI, milk leptin content increased by 45.16 (11.146) pg/mL (0.045 ng/mL) in the Kathmandu sample and by 20.558 (6.745) pg/mL (0.021 ng/mL) in the Nubri sample. There was no significant correlation between maternal BMI and milk adiponectin content for either group (Table 2; Figure 3). However, in the Nubri sample, maternal altitude of residence was a significant, positive predictor of milk adiponectin ($B = 529$ (209) pg/mL per 304 metres [0.529 (0.209) ng/304 m]; 95% CI); over the elevation change, this worked out to an increase of 2.54 ng/mL of milk for mothers in the highest altitude village compared to mothers in the lowest altitude village.

Analyses for the association between these milk hormones and infant WAZ were independent for each group. Mean WAZ were significantly different between Kathmandu and Nubri infants ($p < 0.000$); the differences became greater with increasing infant age (Figure 4). Older infants and toddlers from Nubri had much lower WAZ compared to younger infants, suggesting age associated loss of WAZ among infants from Nubri detectible in these cross-sectional data. By comparison, there were only modest, non-significant declines in WAZ among infants from Kathmandu. There was no significant difference in infant illness frequency between the rural high altitude and urban lower altitude Tibetan samples, although high altitude infants did have slightly higher rates of current illness (31.8% Kathmandu infants vs 38.5% high altitude infants, $p < 0.412$). Nubri infants did have significantly higher rates of coughing and cold-like symptoms (35.7% Nubri vs 20.3% Kathmandu, $p < 0.021$), although we cannot rule out that chronic smoke exposure in the Nubri, but not the Kathmandu infants, may underscore these differences. There were also no significant differences in diarrhoeal illness between the two groups, although again it was more common in the Nubri sample (21.4% Nubri vs 14.5% Kathmandu, $p < 0.145$).

There were no significant associations between milk adiponectin or leptin and WAZ in infants and toddlers from Nubri (Table 3). However, milk adiponectin and leptin had

significant, inverse associations with WAZ for infants and toddlers from Kathmandu (Table 3; Figures 5 and 6). The associations for infants living in Kathmandu persisted after adjustment for infant age, nursing frequency, milk volume and maternal BMI (Table 3). Infant sex was tested as a predictor, but was not significantly associated with either WAZ or milk hormone content.

Discussion

Milk leptin

There were significant differences in mean milk leptin between the two samples of Tibetans (rural high altitude and urban). Maternal BMI was significantly associated with milk leptin in both samples, as has been reported consistently by prior studies. In circulation, leptin is always positively associated with adiposity. In all populations studied to date, milk leptin content is positively associated with both maternal adiposity and her circulating levels of leptin (Casabiell et al., 1997).

Mothers in Kathmandu have a much stronger association between BMI and milk leptin than mothers in Nubri; and this may be underscored by the differences in workload, pathogen exposure and cold stress experienced by the two groups. Outside of maternal BMI and postpartum breastfeeding duration (infant age), few predictors of milk leptin have been identified by prior studies. There are no data from the literature supporting a link between either maternal diet or activity level with either milk or circulating leptin levels (Butte et al., 1997; Casabiell et al., 1997; Karatas et al., 2011), although both have been shown to influence circulating levels of leptin in non-pregnant, non-lactating women (Butte et al., 1997; Casabiell et al., 2001). If such factors influence the hormonal content of human milk is unknown. Additionally, milk leptin may also be derived from mammary synthesis (Bonnet et al., 2002), which may further complicate associations between maternal adiposity and milk leptin content.

There were no significant associations between milk leptin and infant WAZ in the sample of infants from Nubri, before

Table 2. Regression models testing for an association between maternal BMI and milk hormones. Maternal BMI was a significant predictor of milk leptin in both groups. There were no significant associations between maternal BMI and milk adiponectin.

	Unadjusted BMI					Adjusted BMI					Secondary predictors	
	B (SE)	95% CI	p-value	R ²	AIC	B (SE)	95% CI	p-value	R ²	AIC		
Leptin (ng/mL)	Kathmandu	45.162 (11.146)	22.796–67.526	<0.001	0.23	766.61	34.059 (11.850)	10.205–57.913	<0.006	0.14	722.02	Infant age, maternal parity, milk volume, nursing frequency
	Nubri	20.558 (6.745)	7.064–34.051	<0.003	0.12	770.99	22.685 (7.361)	7.932–37.437	<0.003	0.16	760.28	Infant age, maternal parity, milk volume, nursing frequency, altitude
Adiponectin (ng/mL)	Kathmandu	0.0639 (0.088)	–0.099–0.227	<0.436	0.001	881.1	0.057 (0.096)	–0.136–0.252	<0.552	0.001	817.23	Infant age, maternal parity, milk volume, nursing frequency
	Nubri	0.034 (0.121)	–0.208–0.276	<0.780	0.001	1226.49	–0.066 (0.16)	–0.321–0.187	<0.601	0.12	1185.08	Infant age, maternal parity, milk volume*, nursing frequency, altitude**

* $p < 0.05$; ** $p < 0.01$

or after adjustment for infant age. However, milk leptin was significantly and inversely associated with infant WAZ in infants younger than 1 year of age living in Kathmandu. In a previous study also looking at milk leptin and infant WAZ in infants from the developing world (Philippines), with characteristic WAZ decline in the first few years of life, we reported a similar finding: milk leptin is only associated with WAZ in infants less than 1 year of age, not in older toddlers (Quinn et al. 2015). These associations had not been previously reported, probably because most prior studies limit nurslings to less than 1 year of age. Milk leptin was also not associated with infant BMI for age z-score or weight-for-length z-score in either group, although this has been reported for Western samples (Miralles et al., 2006).

There was also no association between infant age and WAZ for infants living in Kathmandu, while WAZ declined with infant age for Nubri infants (Figure 4). These age-related declines in WAZ seen in Nubri infants are likely indexing greater ecological stressors, such as chronic cold stress, under-nutrition or pathogen burden and may contribute to the independence of WAZ from leptin in this group. Two alternative explanations may be considered: first, as this is a cross-sectional study, it is unable to detect the association between milk leptin content and infant growth, as change in milk leptin levels over the course of lactation may be important—infants receiving less milk leptin may have increased weight gain compared to infants receiving more leptin that will be undetectable in a cross-sectional sample. Second, it may be that, in ecologically stressed populations, such as the sample from Nubri, other physiological effects of leptin may be more important. These may include changes to allocation of lean vs fat mass—and such changes in individual tissue types may not be detected at the macro-level of infant weight. Lastly, it is well known that leptin has pleiotropic effects throughout the body. In infants with chronic environmental stress, milk leptin may be important in promoting immune function, gastrointestinal maturation and barrier integrity and may promote specific types of tissue growth—such as white adipose tissue—that may serve as an energy buffer against nutritional shortfalls during acute periods of cold, low nutritional intakes or infection.

Infants produce their own leptin, but it is well established that the leptin in milk enters and is active in neonatal circulation (Casabiell et al., 1997). It is thought that this milk-borne leptin explains the lack of a significant drop in circulating infant leptin in female infants around 4 months post-partum; as the drop is present in all males and formula-fed females (Treviño-Garza et al., 2010). Leptin was originally identified as an energostat for the body, with the idea that leptin may be a kind of ‘life history hormone’. In this interpretation, leptin levels will signal to the body how much energy is generally available and, thus, how much energy can be invested in individual systems, such as growth or maintenance. Prentice et al. (2002) have called leptin ‘the starvation hormone’ based on its increased sensitivity to changes in body composition and energy availability at lower levels, with an overall plateauing of the effect at higher concentrations.

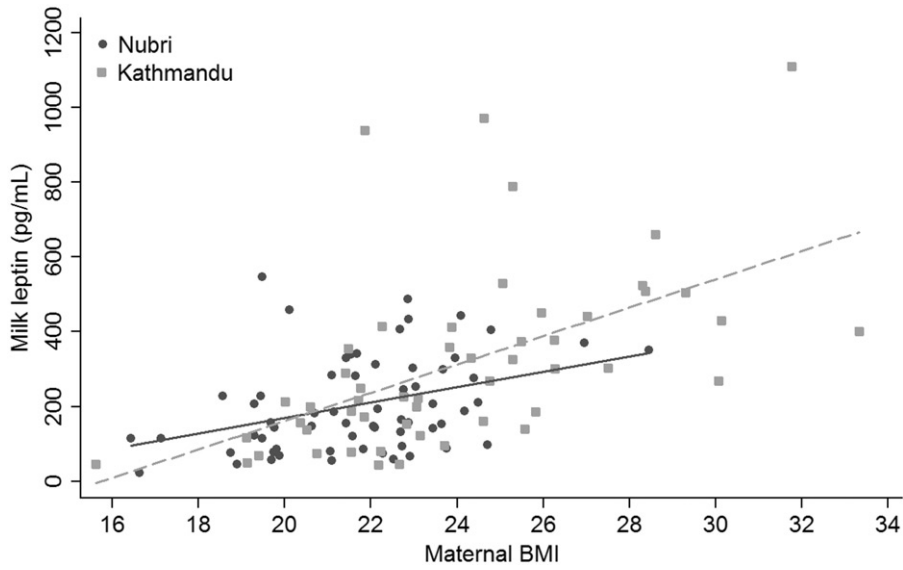


Figure 2. Milk leptin concentrations by maternal BMI for mothers living in Nubri (dark grey) and Kathmandu (light gray). Units here are pg/mL; units in text are ng/mL.

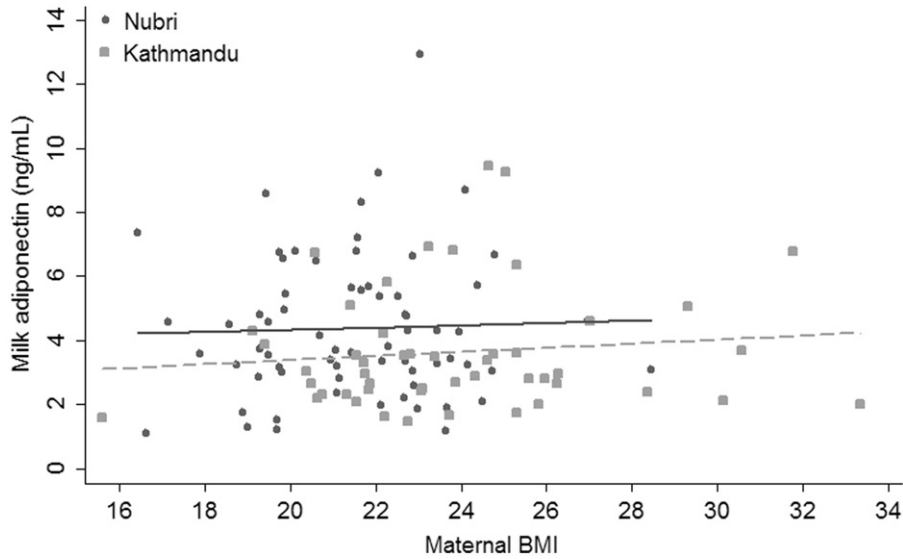


Figure 3. Milk adiponectin concentration by maternal BMI for mothers living in Nubri (dark grey) and mothers living in Kathmandu (light grey). There was not a significant association between maternal BMI and milk adiponectin in either group.

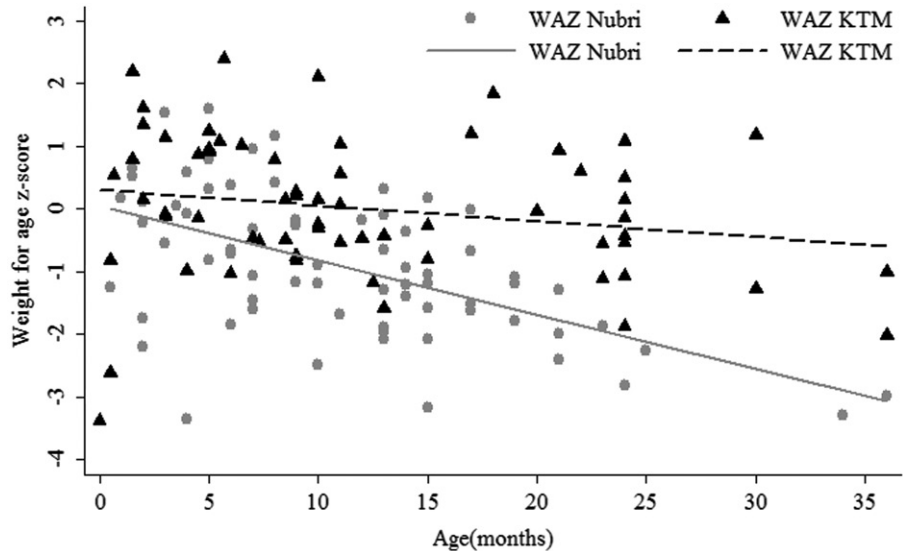


Figure 4. Weight for age-score using the WHO standards by infant age for infants living in Nubri and Kathmandu.

Table 3. Regression models testing for an association between milk hormones and infant WAZ, before and after adjustment for secondary predictors. Only infant age and altitude were predictors of infant WAZ in the Nubri sub-sample; there were no associations between age and WAZ in the Kathmandu sub-sample.

	Unadjusted Leptin					Adjusted Leptin					
	B (SE)	95% CI	p value	R ²	AIC	B (SE)	95% CI	p-value	R ²	AIC	
WAZ	Kathmandu	-0.001 (0.0004)	-0.001 to -0.0007	0.035	0.06	161.57	-0.001 (0.005)	-0.002 to -0.0002	0.020	0.06	160.01
	Nubri	0.001 (0.001)	-0.001 to 0.003	0.251	0.01	194.71	0.001 (0.001)	-0.0007 to -0.0001	0.260	0.33	173.07
WAZ	Unadjusted Adiponectin										
	Kathmandu	-0.0002 (0.0001)	-0.0004 to -0.0008	0.004	0.14	147.10	-0.0003 (0.0001)	-0.0005 to -0.0001	0.003	0.14	187.02
Nubri	-0.006 (0.0064)	-1.333 to 0.122	0.930	0.001	212.31	-0.019 (0.052)	-0.124 to 0.085	0.770	0.38	180.80	
Leptin (ng/mL)	Kathmandu WAZ										
	Adiponectin (ng/mL)	-0.0004 (0.0005)	-0.001 to -0.006	0.396	0.15		-0.0007 (0.009)	-0.001 to -0.002	0.440	0.37	
	Age (months)	-0.234 (0.094)	-0.424 to -0.045	0.016			-0.028 (0.053)	-0.139 to 0.081	0.605		
Age (months)	Nubri WAZ										
		-0.009 (0.020)	-0.049 to 0.032	0.663			-0.088 (0.015)	-0.119 to 0.058	0.00		

Milk adiponectin

Mothers living in the Nubri Valley had significantly higher milk adiponectin compared to mothers living in Kathmandu ($4.3 \pm \text{ng/mL}$ vs $3.6 \pm \text{ng/mL}$), despite lower BMI. There was no significant association between maternal BMI and milk adiponectin in either group, similar to findings reported for prior studies of maternal BMI and milk adiponectin (Dundar et al., 2010; Ley et al. 2012; Martin et al., 2006; Newburg et al., 2010).

Infant age was also not a significant predictor of milk adiponectin in either group. For mothers living in Kathmandu, there was no association between milk volume and milk adiponectin. However, for mothers living in Nubri, milk volume had an inverse association with milk adiponectin. One other factor—altitude of village residence—was also a significant predictor of milk adiponectin. There was a positive association between maternal altitude of residence and milk adiponectin. Mothers living above 3050 metres had significantly higher mean adiponectin compared to mothers living below 3050 ($p = 0.04$).

At present, there are no data available on circulating adiponectin levels among high altitude adapted populations—instead, available research has been concentrated on transient exposure to high altitude in mountaineers and climbers. In these groups, rapid ascent is associated with increased plasma adiponectin (Smith et al., 2011), but this appears to be a short-term adaptation—those with longer stays at high altitude do not show increased plasma adiponectin levels compared to their own baseline measurements taken at sea-level (Barnholt et al., 2006); all studies were conducted in small samples of non-high altitude adapted males. It is unknown if altitude may influence circulating adiponectin levels in high altitude adapted populations or what the potential correlations between maternal adiponectin and milk adiponectin may be in high altitude adapted and living populations.

There was no association between milk adiponectin and infant WAZ among infants living in the Nubri Valley (Figure 6). However, for infants living in Kathmandu, there was a significant, inverse association between milk adiponectin and infant WAZ (Figure 6). For each addition ng/mL of adiponectin in milk, infant WAZ decreased by 0.28 z-scores; Table 3 reports coefficients for pg/mL of adiponectin in milk. These declines are similar to those reported for Mexican populations with good nutritional status and minimal weight for age z-score loss with age (Brunner et al. 2015; Woo et al., 2012). Overall, among infants living in Kathmandu, there was no age-associated loss in weight for age z-score and no measured maternal physiological characteristic was associated with milk adiponectin.

In Nubri, while maternal altitude of residence and milk volume were both predictors of milk adiponectin, there was no significant association between milk adiponectin and infant weight for age z-score. Instead, age and altitude were the primary predictors of WAZ. For each 1 month of age, infants and toddlers in Nubri lost 0.08 z-scores when all other predictors were held constant, with age explaining 21% of the variation in WAZ and associated with declining WAZ. There was a positive

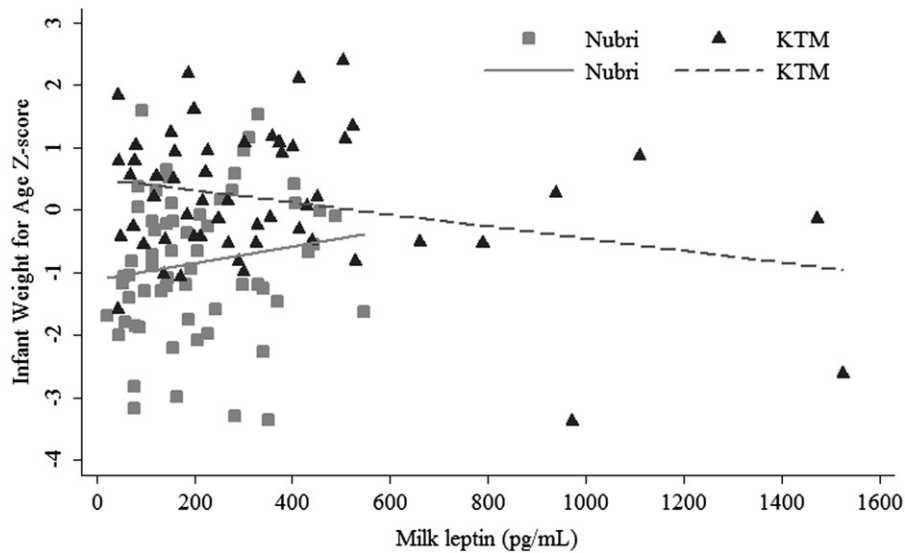


Figure 5. Lack of associations between milk leptin and infant WAZ in the two groups.

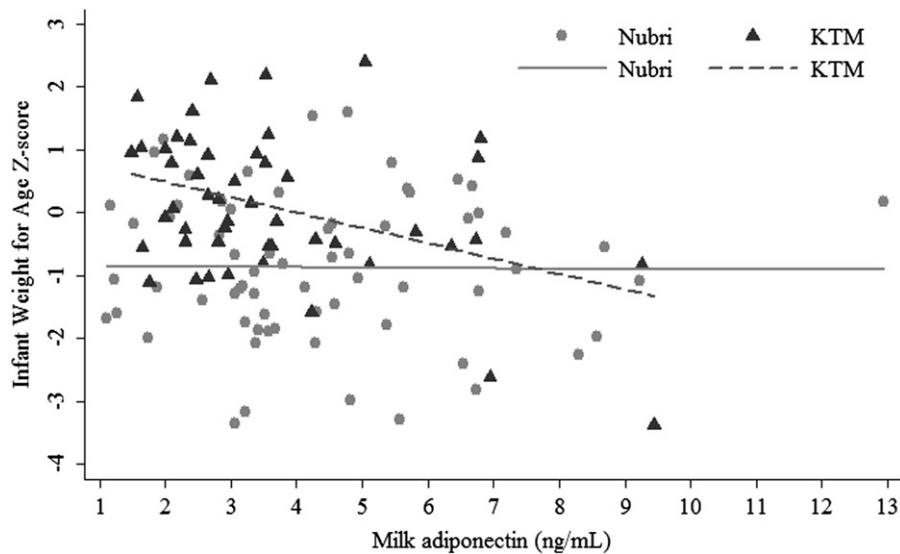


Figure 6. Associations between milk adiponectin and infant WAZ in the two groups; there was no association between milk adiponectin and infant WAZ in Nubri but there was a negative association in the Kathmandu sample.

association between altitude of residence and WAZ in this sample, with infants living above 3050 metres (10 000 feet) having higher mean WAZ compared to infants living at lower altitudes in Nubri; usually WAZ declines with altitude (Beall, 1981; Weitz et al., 2000, 2004; Dang et al., 2004, 2008; Harris et al., 2001). Other ecological factors besides altitude appear to be contributing to the lower weight for age z-scores seen in older infants in Nubri. One explanation for the lack of association between milk hormones and infant weight for age in infants from Nubri may be an environmental override of these associations—that is, ecological pressures that limit infant growth may mask or override maternal ecological signalling.

High altitude growth and milk hormones

It has been well established that Tibetan infants living at high altitude have increased prevalence of wasting and stunting

compared to low altitude living populations (Beall, 1981; Dang et al., 2004, 2008; Harris et al., 2001; Weitz et al., 2000, 2004). Age-associated increases in stunting and wasting are commonly reported, with infants and children living above 4000 metres at highest risk (Dang et al., 2004; Weitz et al., 2000). Here, we had no infants regularly living above 4000 metres, although families living in the two highest villages do have seasonal, short-term migration to these higher altitudes from June–July, meaning that the majority of young infants in the study had not lived at these altitudes. In the sample from Nubri, however, the greatest risks of stunting and wasting were found in the lower portion (below 3000 metres) of the Nubri Valley; the exact opposite of usual patterns reported for high altitude associated growth retardation.

Several factors may have contributed to these differences in weight for age z-scores besides milk composition. There may be survivor bias in the lower Nubri sample, as mortality

rates increase as altitude decreases. However, it seems likely based on these data that ecological pressures, especially those related to pathogen exposure, may be important contributors to the patterns of growth faltering visible in this population. If infants regularly have high pathogen exposure and frequent illness, this may reduce the energy available for growth as the energy will be reallocated to support immune function.

In some ecological contexts, environmental stress can be so great it masks/overrides the underlying biological association because other aspects of the pleiotropic effects of these hormones may be more important for infant survival. The physiological responses may vary—in some contexts, this may present as accelerated growth (weight gain), in others as reduced growth and in some populations there may be no detectable associations. In such contexts, the associations may be experienced as increased survival or possibly in tissue specific physiological changes that cannot be measured at the macro-level of weight or BMI. Adiponectin in milk may promote gut function, protection against infectious pathogens, while leptin in milk may promote immune function in the neonate. Alternatively, leptin and milk adiponectin in milk may promote specific types of tissue growth at the expense of others. For example, if adipose tissue were promoted over lean tissue, this may result in lower overall weight gain despite higher adipose tissue mass. Such prioritisation of body fat, it has been argued, may be an important adaptation to weaning stress (Kuzawa, 1998), increased pathogen exposure associated with motor development/independent locomotion (Wiley & Pike, 1998), seasonal food shortages (Wells, 2012) or other environmentally linked nutritional shortfalls (Wells, 2010). During these stressors, stored fat may be a buffer against starvation, important for energetically expensive brains (Kuzawa, 1998). Finally, we cannot discount certain selective pressures that may be unique to high altitude populations, such as chronic cold stress where additional adipose tissue may serve as an ecological thermal buffer or seasonal food shortages after weaning (Wells, 2000, 2012).

In many populations, external factors requiring increased metabolic investment may override hormonal signals in milk. Infants and toddlers living in Nubri likely have less energy available for growth given high infectious disease burdens and thermal stress and, consequently, these factors obscure associations with milk-borne hormones.

Conclusions

The lack of association between these hormones and infant weight for age z-score may be a remnant of the cross-sectional nature of this sample; however, the presence of the association in the Kathmandu group suggests that the cross-sectional nature of the data alone may not explain the lack of association. Rather, it seems likely that the Kathmandu group may have a reduction in ecological stressors, especially those related to infectious disease burdens. This may suggest that the characteristic associations between milk hormones and infant weight for age may be linked to a reduction in overall ecological pressures such as infectious disease. These

associations then, are part of a biological optimum and may not be present in populations with chronic under-nutrition, nutritional stress and high infectious disease burdens where these pressures obscure such associations. In highly stressed populations, the associations between milk metabolic and growth promoting hormones may be highly variable or not detectable, reflecting the diversity of biological functions of these hormones for neonatal development.

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Declaration of interest

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