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ORIGINAL ARTICLE

Evolution of the circadian profile of human milk amino acids during breastfeeding

Cristina Lucía Sánchez[1, 2], Javier Cubero[3], Javier Sánchez[4], Lourdes Franco[2], Ana Beatriz Rodríguez[2], Montserrat Rivero[5], Carmen Barriga[2]

[1]Translational Neuroscience in Psychiatry and Neurology, Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, RWTH Aachen University, Germany
[2]Chrononutrition Laboratory, Department of Physiology, Faculty of Science, University of Extremadura, Spain
[3]Health Education Laboratory, Experimental Sciences Education Area, University of Extremadura, Spain
[4]Metabolism Laboratory, Perpetuo Socorro Hospital, Servicio Extremeño de Salud (SES), Badajoz, Spain
[5]Scientific Division Manager, Ordesa Group, Barcelona, Spain

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Summary
Human milk is a living fluid that changes with time, composition and volume. Circadian rhythms regulate a variety of biological processes in living organisms; and perhaps the most evident function is the sleep-wake cycle. The aim of the present study was to evaluate the circadian rhythm of breast milk amino acids and their evolution throughout the breastfeeding period. Human breast milk samples from 77 donors were collected every 3 hours over a 24-h period. The rhythmicity of the amino acids was determined by cosinor analysis. Colostrum samples showed no circadian rhythm in most amino acids except tryptophan. However, daily variations were observed in tryptophan and methionine at transitional phase, according to the newborn's pattern of intake every 3 hours regardless of whether it is day or night. During the last stage (mature milk), when breast milk has fully stabilized, most amino acids showed a circadian rhythm. In conclusion, breast milk should be given to the baby at the same time of day it is expressed. Thus, the baby would be adjusting its circadian pattern in harmony with his environment (day/night), which is crucial for the proper functioning and synchronization of all systems in the human body.

Key words: amino acids; day/night; circadian rhythm; human milk; tandem mass

INTRODUCTION

Breast milk and breastfeeding have to be considered as the referent or "gold standard" of infant feeding during the first six months of life (WHO 1985). Breast milk is not a static biological fluid but, rather, it is a dynamic fluid that changes not only over the course of the period of lactation, but also during the day, and even during the same session of nursing, and can be influenced by the mother's own diet (Sánchez et al. 2008, 2009).

The temporal variation in its composition (inter- and intra-individual) has been widely studied. The principal focus has been on its lipid profile (Lammi-Keefe et al. 1990, Lubetzky et al. 2006) and its protein and amino acid content (Clark et al. 1987, Shubat et al. 1989, Catarru et al. 2003, Hernell and Lodernall 2003, Yamawaki et al. 2005, Piccione et al. 2008).

✉ Cristina Lucía Sánchez López, Department of Physiology, Faculty of Science, University of Extremadura, Av. Elvas s/n 06006 Badajoz, Spain
crissanchez@unex.es
☎ + 34 924 289 388
+ 34 924 289 388

The first findings about daily variations in breast milk composition were performed to derive, within the variability of the milk itself, the most appropriate time of day to take the analyte value such as the reference value (Hall 1979, Krebs et al. 1985, Thomas et al. 1986).

Previous studies have demonstrated that breast milk protein levels are greatest in the colostrum, and that they decline in transitional milk to a lower level in mature milk, according to the physiological demand of the newborn. Therefore, associated changes with this content have also been observed; in particular, breast milk protein content is higher during daylight hours than the night period (Sánchez et al. 2011). Other authors have also reported such circadian variations in different components of breast milk, where values may differ between day and night. This is the case for some minerals, such as calcium and magnesium, trace elements, such as zinc (Karra and Kirksey 1998), copper, and iron (Picciano and Guthrie 1976), for some micronutrients, such as lactose, which has minimum values at 17:00 h (Viverge et al. 1986), and for the essential amino acid tryptophan which shows a nocturnal acrophase (Cubero et al. 2005). All these compounds are crucial for the optimal growth of the newborn.

The regulation of the sleep/wake cycle is complex, and involves many neurochemical transmission systems; such as acetylcholine, dopamine, norepinephrine, serotonin, histamine, hypocretin, and orexin, to maintain states of activity, i.e., they are promoters of wakefulness (Murillo-Rodríguez et al. 2009). The concentration of these neurotransmitters is higher during daylight mainly in the brain stem and hypothalamic region, and the variation may be reflected in peripheral fluids such as breast milk (Hunsley and Palmiter 2004). Other neurotransmitters such as gamma-aminobutyric acid (GABA) and serotonin exert their neuromodulatory action on sleep in some brain regions such as the hippocampus and the raphe nucleus, respectively, and their maximum neuroactivity occurs during the night (Reis et al. 2009).

This neural modulation is thought to be present in breast milk, thereby influencing the newborn‘s sleep/wake rhythm through precursor amino acids whose levels may vary throughout the day depending on whether the infant is asleep or active.

In this context, the goal of the present study was to evaluate the circadian rhythm of the breast milk amino acids and their evolution throughout the breastfeeding period, in order to elucidate the importance of these components in regulating the circadian sleep-wakefulness function.

MATERIALS AND METHODS

Subjects

Breast milk samples were collected from 77 breastfeeding women (32±5 years old, 70.8±11.6 kg, 1.65±0.06 m, and body mass index 26.1±4.3 kg/m²). Donors were recruited at the Perpetuo Socorro Hospital (Servicio Extremeño de Salud, S.E.S., Badajoz, Spain). During collection period, the study subjects took no drugs that could affect their amino acid levels. Breastfeeding women at all breast milk stages were asked to participate in the study. All of them received verbal and written information about the methods of the study and they signed an informed consent form before participation. Protocols for this study were approved by the Ethical Investigation Committee from the University of Extremadura (Spain).

Samples

Foremilk samples were collected prior to any feeding from either the left or the right breast into sterile polystyrene tubes (5 ml). Repeated samples from each mother were collected every 3 hours over a 24-h period, regardless of the necessity for feeding, for a total of 6–8 per donor. As the focus of this study was to characterize the circadian rhythm of amino acids in breast milk within each stage, donors were not required to provide samples longitudinally. However, 6 women voluntarily provided milk during all the stages, whereas 22 participants provided samples during 2 stages. The collection campaign lasted from January to December 2008. Breast milk samples were immediately aliquoted and stored at –80 °C until processing.

Extraction of the amino acids

The technique of Yamawaki et al. (2005) was followed with certain modifications. Aliquots of 1 ml of each sample were de-fatted with 0.5 ml of diethyl ether (Sigma-Aldrich). The supernatants were collected, discarding the fatty halo, followed by hydrolysis with 1 ml of 6N HCl (Sigma-Aldrich) for most of amino acids, and 0.75 g of BaOH (Sigma Aldrich) and 1.75 ml of MilliQ water for tryptophan (Cubero et al. 2005). After gentle mixing, the aliquots were allowed to stand for 22 hours at 110 °C, and then filtered through a 0.45 µm membrane filter (Millex, Millipore, USA) to remove the ash before assay.

HPLC-ESI-MS/MS analysis

The samples were assayed using a Waters 2795 Alliance HT HPLC (Milan, Italy) coupled to a Micromass Quattro Ultima mass spectrometer (Milan, Italy) with an ESI (Electrospray Ionization)

source, together with an Agilent Zorbax Eclipse AAA C18 column (3.0 mm × 150 mm × 3.5 micron) for the amino acid analyses. The liquid chromatography–tandem mass conditions were as follows: column temperature 80 °C; source temperature 80 °C; desolvation gas flow 650 l/h; cone gas and voltage 0 l/h and 55 V, respectively; and capillary voltage 3.50 kV (Sánchez et al. 2012).

Chronobiological analysis

A chronobiological analysis (Berger 2011) of the data was performed using the Ritme© for Windows software package. The rhythmicity of each amino acid was studied by cosinor analysis (Halberg et al. 1967). The sinusoidal function used for the fit was:

$$y(t) = M + A \times \cos [(2 \times \pi/\tau) \times t - \varphi]$$

where y(t) is the value of the cosine function at time t, M is the mean level of oscillation or MESOR (acronym for Midline-Estimating Statistic Of Rhythm, the mean value about which the oscillation occurs equal to the arithmetic mean of equidistant data covering a whole number of cycles), A is the amplitude (measure of the extent of a rhythmic change in a cycle as estimated by the sinusoidal function that best fits the data), the angular frequency is $\omega = 2 \times \pi/\tau$ where π is the number pi and τ is the period (24 hours in our case), and φ is the acrophase (a phase angle measuring the timing of the peak activity, expressed as the lag from a reference time to the crest time of the best fit sinusoidal function). A cosinor analysis thus finds the best-fitting sinusoidal function by estimating three parameters: mesor, amplitude, and acrophase.

Using the cosinor analysis, we determined the 95% confidence intervals of the MESOR, amplitude, and acrophase. When this interval contains the value 0, one possibility – that amplitude is 0 – cannot be rejected, so that the existence of a rhythm is not statistically significant. This is equivalent to testing whether the null hypothesis of zero amplitude is rejected at 2α level of 0.05.

The confidence intervals of the acrophase show whether there are significant differences between the acrophases of different variables. In particular, if two confidence intervals overlap, the possibility that the two acrophases are equal can not be discarded.

RESULTS

Colostral stage (<5 days post-partum)

Table 1a, b summarizes the mean concentrations of each amino acid in mg/dl during the colostrum period. To facilitate understanding of the data, the table is presented in two parts: daytime (09:00 to 21:00, Table 1a) and night-time (21:00 to 09:00, Table 1b).

Table 1a. **Concentrations of amino acids** (mg/dl) **during the colostral stage at 09:00–18:00** (n = 31).

	9:00	12:00	15:00	18:00	mean	SD
Alanine	63.92	58.83	78.40	61.45	55.93	10.39
Arginine	58.77	54.88	73.68	58.04	53.33	10.27
Aspartic acid	111.52	105.26	130.55	109.28	97.75	18.32
Citrulline	3.72	3.98	5.05	4.20º	4.36	2.49
Glutamic acid	228.41	219.73	271.05	232.80	222.38	36.76
Glycine	34.33	33.24	42.67	33.60	31.02	7.70
Hystidine	28.64	27.24	34.53	29.32	25.95	6.00
Leucine	97.26	92.60	122.98	102.08	96.95	18.21
Methionine	30.62	24.51	36.49	31.31	30.04	12.55
Ornitine	6.51	6.04	7.69	6.91	7.48	2.95
Phenylalanine	50.04	46.76	61.94	50.08	45.33	8.47
Proline	123.14	113.19	143.84	119.82	114.46	21.72
Serine	59.93	56.35	69.94	59.92	53.40	10.50
Tryptophan	34.74	34.69	34.52	34.61	34.64	0.37
Tyrosine	45.00	42.02	55.47	45.56	41.37	7.79
Valine	61.14	57.92	77.87	64.85	60.37	12.20
Taurine	73.66	69.93	67.65	72.15	71.81	11.90

Table 1b. **Concentrations of amino acids** (mg/dl) **during the colostral stage at 21:00–06:00** (n = 31).

	21:00	24:00	3:00	6:00	mean	SD
Alanine	59.25	61.62	50.39	59.67	57.73	11.72
Arginine	54.49	56.37	39.20	66.61	54.17	13.46
Aspartic acid	102.44	108.61	76.08	116.32	100.86	22.59
Citrulline	3.80	4.53	3.38	5.41	4.28	1.98
Glutamic acid	210.46	236.81	194.43	249.83	222.88	46.86
Glycine	32.05	32.14	22.02	39.58	31.45	9.72
Hystidine	27.76	28.54	20.27	30.74	26.83	6.35
Leucine	92.48	101.69	87.18	106.68	97.01	24.91
Methionine	27.56	27.56	26.62	34.44	29.05	11.37
Ornitine	7.06	7.21	5.87	9.22	7.34	3.32
Phenylalanine	46.89	49.89	35.71	53.73	46.55	10.65
Proline	110.52	121.62	97.13	131.15	115.11	25.22
Serine	57.94	58.61	39.92	64.93	55.35	11.62
Tryptophan	34.62	34.81	34.76	34.79	34.74	0.33
Tyrosine	41.69	45.45	33.91	47.94	42.25	9.90
Valine	58.20	62.82	53.19	67.49	60.42	13.99
Taurine	71.52	75.39	72.27	70.69	72.46	11.25

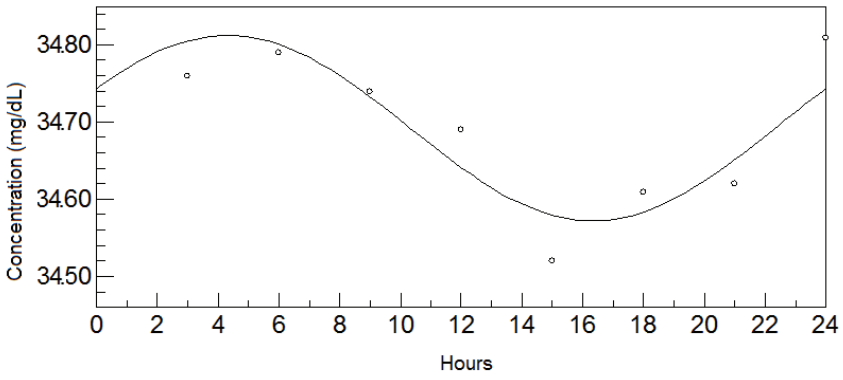

Fig. 1. **24-hours oscillations of tryptophan in colostrum.** This graph depicts the fluctuations of tryptophan levels in colostrum during 24-hours. Circadian rhythm is shown by a sinusoidal curve.

These results show how the amino acids vary during this stage of lactation according to the time of day. However, the Cosinor analysis of the mean values reveal a daily (24-hour) rhythmicity only in the essential amino acid tryptophan (Fig. 1), which perhaps exerts a marked effect on the infant from its first feed after birth.

Transitional stage (6–15 days post-partum)

Table 2a, b presents the daytime (a) and night-time (b) concentrations of the amino acids during the transitional phase. This stage, as its name indicates, is characterized by an intermediate composition between the colostrum and the mature milk. It occurs from days 6 to 15 of lactation.

The amino acid profile varies over the course of the day (Figs 2 and 3). In addition, the concentrations of most of the amino acids decline as nursing advances.

The chronobiological analysis again showed the amino acid tryptophan (Fig. 2) to continue to present a circadian rhythm with acrophase during the night (03:00 approx.). However, another essential amino acid, methionine (Fig. 3), also showed a marked circadian rhythm, with acrophase during the daytime (18:00 approx.). Variations in concentrations at different times of day and the chronobiological data are shown in Tables 2a, b and 4, respectively.

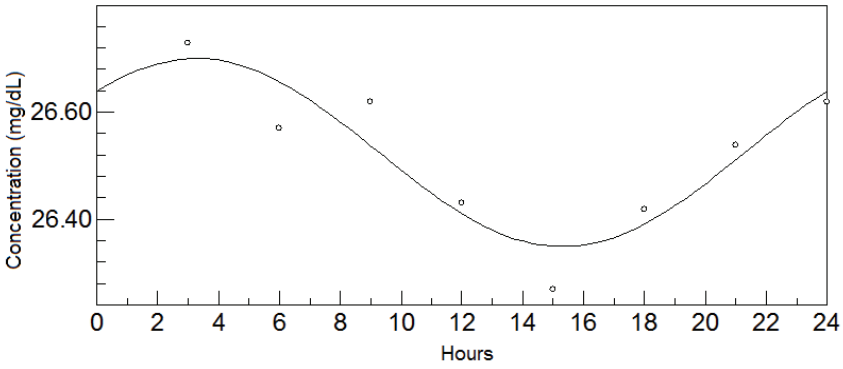

Fig. 2. **24-hours oscillations of tryptophan in transitional milk.** This graph depicts the fluctuations of tryptophan levels in transitional milk during 24-hours. Circadian rhythm is shown by a sinusoidal curve.

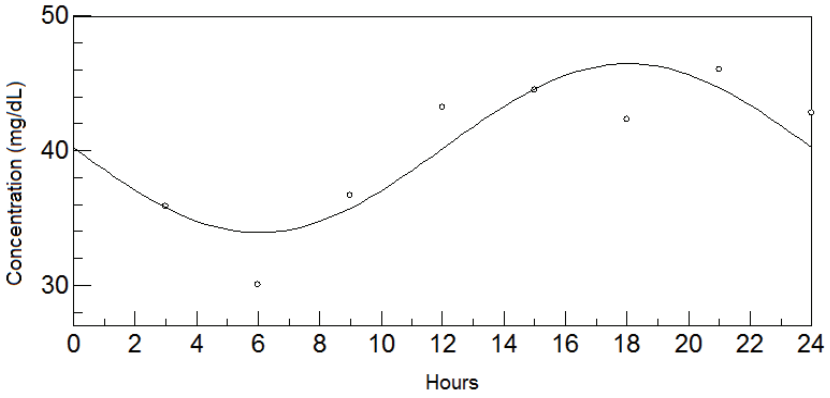

Fig. 3. **24-hours oscillations of methionine in transitional milk.** This graph depicts the fluctuations of methionine levels in transitional milk during 24-hours. Circadian rhythm is shown by a sinusoidal curve.

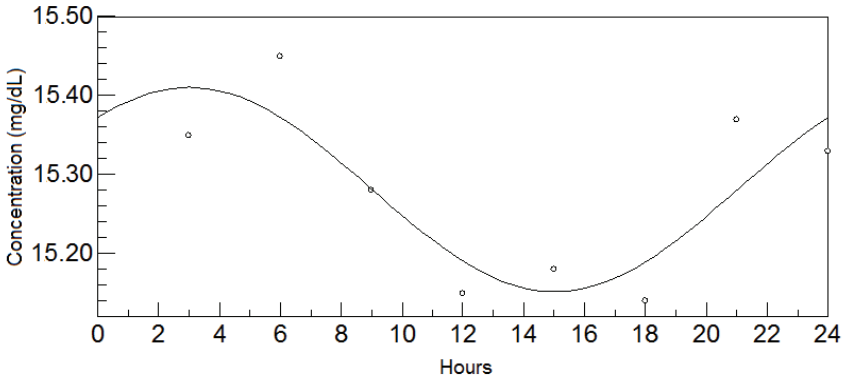

Fig. 4. **24-hours oscillations of tryptophan in mature milk.** This graph depicts the fluctuations of tryptophan levels in mature milk during 24-hours. Circadian rhythm is shown by a sinusoidal curve.

Table 2a. **Concentrations of amino acids** (mg/dl) **during the transitional stage at 09:00–18:00** (n = 34).

	9:00	12:00	15:00	18:00	mean	SD
Alanine	51.04	49.58	48.26	48.01	49.22	11.85
Arginine	44.83	41.01	40.53	39.40	41.44	10.65
Aspartic acid	82.73	86.95	84.33	83.00	84.25	17.45
Citrulline	2.73	3.91	3.33	3.24	3.30	1.81
Glutamic acid	170.88	181.21	175.92	175.00	175.75	35.00
Glycine	30.50	27.16	27.68	26.56	27.97	8.93
Hystidine	32.23	26.44	26.13	25.43	27.56	6.45
Leucine	65.99	69.50	69.92	67.82	68.31	14.42
Methionine	36.68	43.25	44.57	42.34	41.71	11.68
Ornitine	8.26	8.17	8.43	7.57	8.11	5.70
Phenylalanine	36.73	38.25	38.68	36.82	37.62	8.19
Proline	88.64	94.68	93.90	91.78	92.25	19.35
Serine	60.14	55.59	55.52	53.41	56.16	14.34
Tryptophan	26.62	26.43	26.27	26.42	26.44	0.54
Tyrosine	32.65	33.01	32.47	31.32	32.36	7.14
Valine	48.68	51.13	51.31	49.59	50.18	11.81
Taurine	64.93	75.23	79.33	75.96	73.86	19.44

Table 2b. **Concentrations of amino acids** (mg/dl) **during the transitional stage at 21:00–06:00** (n = 34).

	21:00	24:00	3:00	6:00	mean	SD
Alanine	51.12	44.71	45.57	42.07	45.87	8.91
Arginine	41.32	37.86	39.37	36.83	38.84	8.25
Aspartic acid	87.73	82.15	84.69	83.25	84.45	15.68
Citrulline	4.81	3.30	4.73	3.11	3.99	3.08
Glutamic acid	185.94	172.05	178.42	178.11	178.63	37.72
Glycine	27.34	23.62	24.36	22.87	24.55	5.52
Hystidine	27.40	24.30	25.32	23.55	25.14	5.35
Leucine	70.91	66.34	67.34	66.43	67.75	13.35
Methionine	46.10	42.84	35.86	30.07	38.72	10.11
Ornitine	8.80	6.64	5.79	5.63	6.72	3.46
Phenylalanine	39.37	36.19	36.95	35.30	36.95	7.13
Proline	98.25	91.51	89.79	93.10	93.16	19.48
Serine	58.52	50.12	51.37	49.41	52.36	10.04
Tryptophan	26.54	26.62	26.73	26.57	26.62	0.28
Tyrosine	33.54	30.86	31.56	30.18	31.54	6.15
Valine	51.87	47.92	48.60	46.98	48.84	9.23
Taurine	78.75	72.31	71.06	71.55	73.42	13.53

Mature stage (>15 days post-partum)

The results of the assay of amino acids in the mature stage of breast milk are shown during daytime in Table 3a and night-time in Table 3b. At this stage, most components in breast milk are stabilized for the remaining months of lactation as the chronobiological analysis shows. Besides tryptophan and methionine (Figs 4 and 5 respectively), additional amino acids such as aspartic acid (Fig. 6), histidine (Fig. 7), phenylalanine (Fig. 8) and tyrosine (Fig. 9), present circadian oscillations in their concentrations.

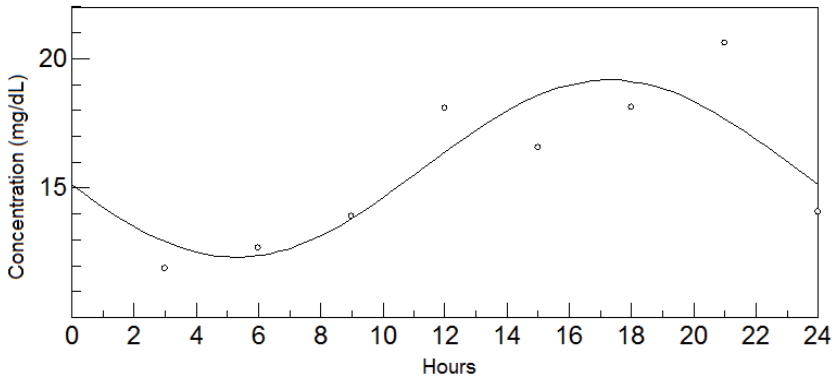

Fig. 5. **24-hours oscillations of methionine in mature milk.** This graph depicts the fluctuations of methionine levels in mature milk during 24-hours. Circadian rhythm is shown by a sinusoidal curve

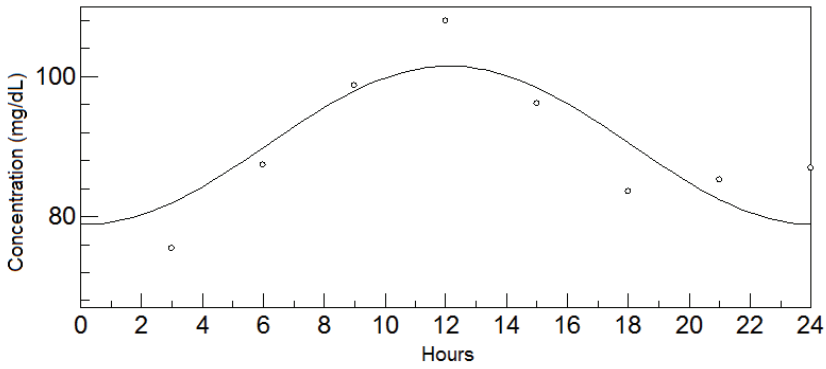

Fig. 6. **24-hours oscillations of aspartic acid in mature milk.** This graph depicts the fluctuations of aspartic acid levels in mature milk during 24-hours. Circadian rhythm is shown by a sinusoidal curve.

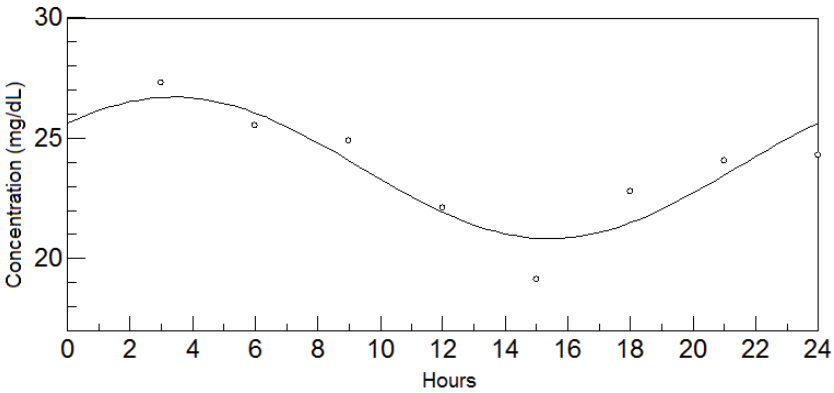

Fig. 7. **24-hours oscillations of histidine in mature milk.** This graph depicts the fluctuations of histidine levels in mature milk during 24-hours. Circadian rhythm is shown by a sinusoidal curve.

Table 3a. **Concentrations of amino acids** (mg/dl) **during the mature stage at 09:00–18:00** (n = 40).

	9:00	12:00	15:00	18:00	mean	SD
Alanine	41.70	44.04	35.15	38.34	39.81	9.56
Arginine	38.93	44.46	34.08	38.16	38.91	11.76
Aspartic acid	98.74	107.99	96.17	83.57	96.62	17.91
Citrulline	6.11	7.55	6.06	5.41	6.28	2.53
Glutamic acid	153.88	167.03	140.86	137.45	149.81	19.88
Glycine	26.84	30.21	28.39	24.65	27.52	5.65
Hystidine	24.90	22.12	19.13	22.81	22.24	3.82
Leucine	67.73	64.91	63.49	60.35	64.12	11.22
Methionine	13.93	18.09	16.57	18.15	16.69	4.83
Ornitine	9.04	9.98	8.69	8.96	9.17	2.72
Phenylalanine	37.24	40.27	37.37	35.53	37.60	8.87
Proline	83.71	92.26	74.06	70.21	80.06	16.42
Serine	48.00	51.24	41.42	44.27	46.23	12.58
Tryptophan	15.28	15.15	15.18	15.14	15.19	0.06
Tyrosine	36.27	36.00	33.34	29.31	33.73	5.70
Valine	54.84	53.44	52.25	49.80	52.58	9.09
Taurine	63.44	64.32	65.06	64.89	64.43	1.63

Table 3b. **Concentrations of amino acids** (mg/dl) **during the mature stage at 21:00–06:00** (n = 40).

	21:00	24:00	3:00	6:00	mean	SD
Alanine	42.32	40.60	38.16	41.03	40.53	7.56
Arginine	39.49	39.85	32.69	36.38	37.11	9.88
Aspartic acid	85.31	86.88	75.54	87.40	83.78	21.11
Citrulline	6.01	6.51	5.72	5.34	5.89	1.61
Glutamic acid	154.29	145.53	139.73	175.02	153.64	29.54
Glycine	23.18	25.32	24.89	26.57	24.99	3.03
Hystidine	24.10	24.33	27.31	25.56	25.32	3.54
Leucine	71.54	68.77	59.92	80.10	70.08	13.21
Methionine	20.62	14.11	11.91	12.72	14.84	2.79
Ornitine	9.31	8.36	9.63	7.79	8.77	3.02
Phenylalanine	34.40	32.07	32.90	35.32	33.67	7.09
Proline	72.54	73.52	71.83	95.78	78.42	18.39
Serine	46.02	46.81	39.31	47.81	44.99	9.83
Tryptophan	15.37	15.33	15.35	15.45	15.37	0.05
Tyrosine	28.59	29.55	30.32	36.47	31.23	6.77
Valine	58.37	52.13	49.54	62.20	55.56	11.41
Taurine	63.99	64.10	63.71	64.68	64.12	2.12

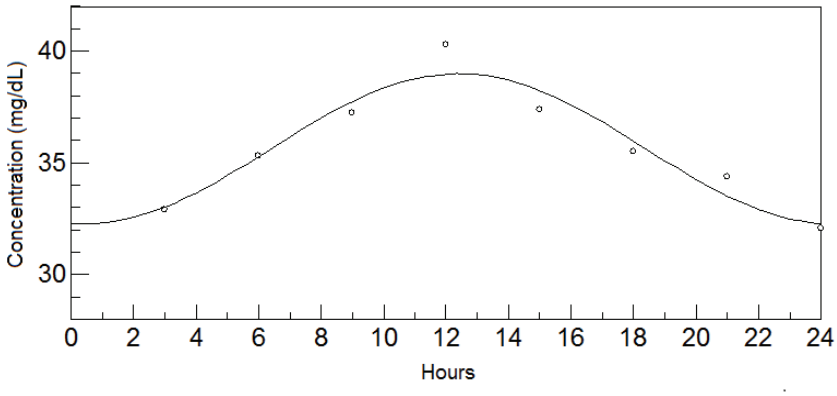

Fig. 8. **24-hours oscillations of phenylalanine in mature milk.** This graph depicts the fluctuations of phenylalanine levels in mature milk during 24-hours. Circadian rhythm is shown by a sinusoidal curve.

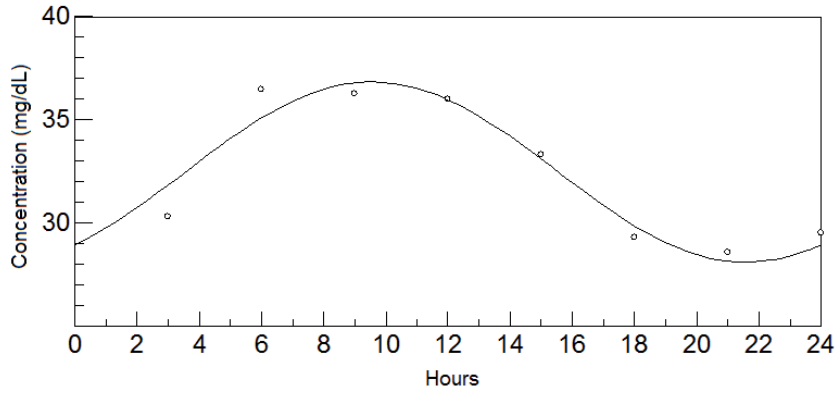

Fig. 9. **24-hours oscillations of tyrosine in mature milk.** This graph depicts the fluctuations of tyrosine levels in mature milk during 24-hours. Circadian rhythm is shown by a sinusoidal curve.

Table 4 lists the chronobiological parameters MESOR, amplitude and acrophase of the amino acids which present circadian rhythms in the different stages of lactation.

DISCUSSION

The results of the present study consistently characterize a circadian rhythm in some amino acids in breast milk throughout the evolution of the breastfeeding period. These results are consistent with those reported previously by other authors (Yamawaki et al. 2005, Piccione et al. 2008). Previously, we found that breast milk from donors in Spain showed strong daily variations in the protein content, suggesting the presence of a regulating mechanism controlled by circadian rhythms in breast milk (Sánchez et al. 2011).

Amino acids are nitrogenous components and one of their main functions is protein anabolism, which is accelerated in newborns whose structural demand is very high. All components analysed in the present work, except for aspartic acid and glycine, are essential for neonates and crucial for their growth and development (Ballabriga and Carrascosa 2001).

The novel contribution of the present work is the idea that amino acids in breast milk change during the course of the day, in particular asking whether the acrophase occurs during the stage of sleep or of wakefulness, in consonance with the neural demand of the newborns. The appropriate timing of the acrophase is a minor consequence; to be quantified with precision, it would be necessary to study a large population affected by a less temporal heterogeneity, i.e., collecting all the breast milk samples at the same time of day and season of the year, which was not feasible for us.

During the colostrum period, breast milk is almost lacking circadian rhythm, reflecting the still premature secretion of the milk, and because the infant's sleep/wakefulness function is not fully established. Furthermore, the intervals between each feeding

Table 4. **Chronobiological parameters (mean ± SD) of the amino acids which showed a circadian rhythm.** The units of the MESOR values and amplitudes are mg/dl. The acrophases are given as times of day (08:00–20:00–08:00 light–dark cycle). * Statistically significant.

Amino acids	COLOSTRAL MILK			TRANSITIONAL MILK			MATURE MILK		
	MESOR	Amplitude	Acrophase	MESOR	Amplitude	Acrophase	MESOR	Amplitude	Acrophase
Tryptophan	34.69±0.05	0.12±0.09	4:19±3:21*	26.53±0.06	0.18±0.06	3.16±2:38*	15.26±0.06	0.13±0.12	3:00±4:24*
Methionine	–	–	–	39.75±2.81	6.36±5.26	17:57±3:43*	15.25±1.79	3.68±3.35	17:19±4:22*
Aspartic acid	–	–	–	–	–	–	89.62±5.80	11.27±10.84	12:08±4:34*
Histidine	–	–	–	–	–	–	23.38±1.04	2.89±1.94	3:14±2:48*
Phenylalanine	–	–	–	–	–	–	35.25±5.85	3.43±1.59	12:33±1:53*
Tyrosine	–	–	–	–	–	–	32.12±0.87	4.54±1.62	9:38±1:23*

are very short and constant. We only observed the beginning of a circadian rhythm for the essential amino acid tryptophan, which was confirmed throughout the subsequent stages.

During the transitional stage, in addition to tryptophan, methionine also showed a circadian rhythm (Sánchez et al 2010). Its acrophase pattern seems to be opposite to the tryptophan pattern, i.e., during the day, in the stage of wakefulness and with a greater likelihood of infant activity.

Finally, it is during the mature stage where the circadian rhythms of the activity-promoting neuroactive amino acids unfold: phenylalanine, an essential amino acid; tyrosine, precursor of norepinephrine and epinephrine (Hunsley and Palmiter 2004, Mitchell and Weinshenker 2010); methionine, an essential amino acid and precursor of acetylcholine (Sugimoto et al. 1964, Chabannes et al. 1984, Sánchez et al. 2010); and aspartic acid and glycine, activity neurotransmitters exhibited an acrophase during wakefulness (Shubat et al. 1989).

Histidine results were unexpected because this amino acid is the histamine precursor – an excitatory neurotransmitter (Monti 1993) – and its acrophase is unforeseen given that it occurs during the period of darkness, i.e., when there is a greater tendency to sleep. A possible reason for this is that this component is a semi-essential amino acid at this stage of life and its endogenous levels may not be influenced by diet (Ballabriga and Carrascosa 2001).

Although the variations in our study were small, they can be significant in terms of neural response. Tryptophan showed a circadian rhythm in all three stages of lactation (colostrum, transitional, and mature milk) and strong evidence has been noted on the action of this essential amino acid on the neurotransmitter serotonin and the hormone melatonin, both of which are well-known sleep-inducing biogenic amines (Hajak et al. 1991, Heuther et al. 1992).

We can summarize that breast milk is a dynamic biological fluid in that its composition is not constant, but evolves throughout the day and over the course of lactation, just as diet evolves during the day and throughout the course of our lives (Ballabriga and Carrascosa 2001).

It could be argued again in defence of breastfeeding compared to artificial feeding because formula milks have a further handicap that has to be considered as it does not hold the synchronization of the neuromodulatory amino acids that breast milk does. This temporal rhythm exhibited in the components of breast milk may give to the infant a physiological advantage in development, including the sleep function, compared with artificial feeding, given that the amino acid content is constant throughout the day.

This daily timekeeping could be driven by transcriptional/translational feedback loops, whereby rhythmic expression of "CLOCK" gene products regulates the expression of associated genes in approximately 24-hour cycles (O'Neill et al. 2011). Recently, a metabolomics study has shown that synthesis and degradation of nucleotides (other nitrogenous components which circadian rhythm has been already demonstrated in breast milk – Sánchez et al. 2008) in the liver are under transcriptional circadian control (Fustin et al. 2012).

Even though some information exists in the field of chronobiology in respect to breast milk, and the chrononutrition "boom" during recent years has increased the awareness of the importance of breastfeeding to newborn health, further research into the formation , composition, and biological effects of human breast milk should be encouraged.

Further studies are also needed to determine the mechanisms of action of the processes responsible for these daily variations in breast milk, even given that the alignment of internal rhythms with the outside world is well-known to be affected by the time of feeding as well as by the light/dark cycle. Perhaps a correlation between the circadian pattern of the breast milk components and the sleeping efficiency of the mothers should be conducted in a future to obtain a better knowledge of these biological processes.

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