

# CHARACTERIZATION OF PROTEINS AND FATTY ACID COMPOSITION IN GALAPAGOS FUR SEAL MILK. OCCURRENCE OF WHEY AND CASEIN PROTEIN POLYMORPHISMS

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**Abstract**—1. Milk proteins of the Galapagos fur seal (*Arctocephalus galapagoensis*) were separated adequately into whey and casein fractions using bovine milk analysis methods.

2. In samples from days 5-30 of lactation 40% of the total proteins were whey and 60% caseins; in mid-lactation, day 150, 25% were whey and 75% casein proteins.

3. Electrophoretic and isoelectric focusing patterns of fur seal whey protein differed widely from bovine patterns, whereas those of caseins were similar.

4. Polymorphisms of fur seal whey and casein proteins were noted and did not seem related to different stages of lactation.

5. C-16 and C-18 fatty acids contributed about 70% of fatty acids; 63% of the total acids in milk fat were unsaturated.

## INTRODUCTION

The milk of marine mammals is unusually high in fat and protein (Jenness, 1974). While many data are now available of gross composition of such milk (Bonner, 1984; Oftedal *et al.*, 1986) little is known about the specific protein and fatty acid composition (e.g. Ackman and Burgher, 1963; Ashworth *et al.*, 1966; Horn and Baker, 1971; Jenness *et al.*, 1981; Pilon and Kelly, 1962). Initially we also began with an analysis of the fat and protein content of the milk of the Galapagos fur seal (*Arctocephalus galapagoensis*) and sea lion (*Zalophus californianus wollebaeki*) and determined how milk composition changes with the age of the pup, and how the measured composition depends on the sampling method (Trillmich and Lechner, 1986). At the same time we began to investigate casein and non-protein nitrogen (NPN) content and tried to characterize proteins by polyacrylamide gel (PAG) electrophoresis. The electrophoresis showed differences in milk protein patterns between fur seal females (Kirchmeier, unpublished).

To determine whether the differences between our samples were due to polymorphisms or to the stage of lactation we took repeated samples from four fur seal females and an additional six mid-lactation samples in March 1987. These samples were analyzed by gel electrophoreses and isoelectric focusing to characterize protein fractions. We also investigated whether the separation methods used for bovine milk produce meaningful results when applied to fur seal milk (Jenness and Sloan, 1970) and obtained information on the fatty acids in the milk fat.

## MATERIALS AND METHODS

### Samples

Milk samples were obtained from Galapagos fur seal mothers (*Arctocephalus galapagoensis*) caught on Fernandina Island, Galapagos. The animals were briefly restrained for milking, injected intra-muscularly with 2 ml of oxytocin (10 units/ml) and samples of 30-60 ml were milked from one teat. Samples were immediately mixed with sodium azide and stored at ambient temperature (*ca* 22°C) before being shipped to Germany where they were deep frozen until analysis. Samples are listed in Table 1.

### Analytical methods

Total nitrogen (TN) and non-protein nitrogen (NPN) were determined as described in Trillmich and Lechner (1986). Total protein nitrogen (TPN = TN - NPN) was determined as ZnSO<sub>4</sub>-precipitated nitrogen. Non-casein nitrogen (NCN) was nitrogen in the supernatant of milk

Table 1. Sources of Galapagos fur seal milk samples. Female numbers and age of pup (in days) at the time of milking

Female	Age of pup	Year of sampling
8	1	1983
11	17	
13	30	
14	3	
15	14	1984
269	1, 4, 12	
271	1, 6, 12	
272	2, 6, 14	
273	1, 5, 15	
1-6/87	<i>ca</i> 150	1987

adjusted to pH 4.7. Therefore, casein nitrogen (CN) = TN - NPN, and whey protein nitrogen (WPN) = TPN - CN. Protein content was calculated by multiplying TPN, CN and WPN by the factor 6.38.

#### Electrophoresis

After fat extraction cellulose acetate gel (CAG) electrophoresis was run for caseins in a phosphate-urea buffer (0.92 g citric acid monohydrate, 3.56 g Na<sub>2</sub>HPO<sub>4</sub>, 360 g urea, 1.5 ml mercaptoethanol per 1000 ml) at pH 7.0 (4°C, 200 V, 3 hr). Whey proteins were run on CAGs in a Tris-glycine buffer (6.06 g Tris, 18.75 g glycine per 1000 ml) at pH 8.6 (4°C, 200 V, 1 hr). Proteins were stained with amido black.

Polyacrylamide gel (PAG) electrophoresis of milk proteins was performed according to application note 306 of the LKB-Instrument GmbH (D-8032 Gräfefing, FRG).

#### Isoelectric focusing (IEF)

Caseins were precipitated from defatted milk at pH 4.7. The precipitate was extracted twice with dichloromethane-water (Krause *et al.*, 1982) and the casein dried with acetone. Whey proteins were isolated by dialysis and lyophilization of the supernatant. Then 4 mg of casein and 1 mg of whey protein were dissolved in 1 ml of 8 mol/l urea and 0.03 mol/l dithioerythritol (DTE) and applied to the PAG.

Alternatively, whole milk was defatted and urea was added to give a final concentration of 8 mol/l; the clear solution was diluted with 8 mol/l urea containing 0.03 mol/l DTE to protein concentrations suitable for isoelectric focusing (IEF). IEF was performed at pH 2.5–8.0 in 4.7% PAGs (260 × 200 × 0.25 mm) in the presence of 7.5 mol/l urea and 10% glycerol. The gel was run at 10°C with 7 W and a maximum voltage of 2500 V for 3.0 hr, including a 30 min pre-run (Krause, 1987; Krause and Belitz, 1985).

Approximately 60 µg of caseins and 15 µg of whey proteins were applied on the anodic side of the gel; the amount of protein in whole-milk samples varied. Proteins were stained with methanolic Servablue G 250 after fixation in 20% trichloroacetic acid. Isoelectric points (pI) of proteins were measured at 10°C with a surface electrode and compared with pI of casein markers. No corrections were made for urea.

#### Gas chromatographic analysis of fatty acids

Fat extraction followed the method of Röse-Gottlieb (Horwitz, 1965). Transesterification of the fatty acids to methyl esters was done according to Hadorn and Zürcher (1971). Capric acid methylester was added as an internal standard. Gas-liquid chromatography (GLC) of the methyl esters was carried out using hydrogen as a carrier gas on a Hewlett-Packard 5790 with a Durabond-wax capillary column of 30 m length (Diethylene glykol phase). The column was heated from 40 to 220°C at a rate of 10°C/min. The results are based on three GLC-runs.

## RESULTS

#### Protein composition and characterization

Isoelectric precipitation at pH 4.7 separated the proteins of the fur seal milk into two well-defined fractions. The precipitated proteins were shown to be caseins as they were sensitive to rennin (EC 3.4.23.4) treatment and only dissolved in urea buffer. The proteins in the supernatant fraction were coagulated by heat treatment, indicating that the fractionation procedure successfully separated the albumins and globulins (whey proteins) from the caseins (caseins have high heat stability).

Table 2. Protein composition of Galapagos fur seal milk. NPN = nonprotein nitrogen (g/100 g)

	Total protein (%)	NPN	Casein (%)	Whey (%)	Female	Age of pup (days)
	12.4	0.45	5.0	7.4	273	1
	7.9	0.26	4.5	3.3		5
	9.4	0.28	6.4	3.0		15
	9.1	0.21	4.9	4.3		6
	9.7	0.32	6.1	3.6	272	14
	10.2	0.15	5.4	4.8	14	3
	9.4	0.07	6.1	3.3	15	14
	11.8	0.12	7.5	4.2	13	30
Mean	9.98	0.23	5.75	4.23		
SD	1.46	0.12	0.99	1.42		
	14.9	0.63	11.6	3.3	1/87	150
	14.6	0.71	10.5	4.1	2/87	150
	14.3	0.80	11.1	3.2	3/87	150
	12.2	0.48	8.1	4.1	4/87	150
	14.0	0.61	10.2	3.8	5/87	150
	13.8	0.73	11.5	2.3	6/87	150
Mean	14.0	0.66	10.5	3.5		
SD	0.95	0.11	1.3	0.7		

Total nitrogen (TN) content of the milk was highest on the first day of lactation in three out of four females which were milked repeatedly. The means and SDs were:  $2.39 \pm 0.33\%$  on day 1,  $N = 4$ ;  $1.85 \pm 0.31$  day 3–30,  $N = 8$ ; ( $P = 0.048$ , Mann Whitney *U*-test). This was mainly due to a higher whey protein and NPN content on day 1. While whey protein remained nearly constant after the first day of lactation (Table 2), casein increased up to day 150 (regression: casein (%) =  $0.035d + 5.32$ ;  $d$  = age of pup in days;  $r = 0.93$ ,  $P < 0.001$ ). Over the first 30 days of lactation the amount of casein in the milk increased by over 50% (Table 2). This fits with the reported increase in milk Ca-content with age of the pup (Trillmich and Lechner, 1986). By day 150 of lactation caseins had roughly doubled. In milk samples from days 5–30 of lactation, caseins contributed  $61.9 \pm 5.5\%$  (mean  $\pm$  SD) and whey proteins  $38.1 \pm 5.5\%$  of the milk proteins, giving a ratio of roughly 1.6:1, by day 150 this had changed to 75% caseins and 25% whey, 3:1 (Table 2).

IEF showed that the fur seal's casein fractions were similar to those of cow's or other ruminants' milk (Fig. 1). However, since the amino acid sequence of fur seal caseins was not determined, true homology could not be established. Therefore casein fractions were provisionally assigned roman numerals; arabic numbers were assigned to the protein components of each fraction in order of increasing mobility of pI's as recommended by Eigel *et al.* (1984). Latin letters were used for obvious polymorphisms. Where similarity seemed clear the corresponding name of the bovine milk protein fraction is mentioned.

#### Caseins

Cn-I (similar to alpha-s1-casein) had a pI of 4.9 and the second fraction—Cn-II ( $\beta$ -Cn like)—was a variable group with up to four zones with pI's of 5.5, 5.55, 5.6, and 5.7. Cn-III with a pI of around 6.0 (Figs 1 and 2) seemed to be a k-casein, judging from its sensitivity to rennin (EC 3.4.23.4). The minor casein fraction with pI's above 6.3 (Fig. 1) were collectively labeled Cn-IV and may be similar to the bovine  $\gamma$ -caseins.

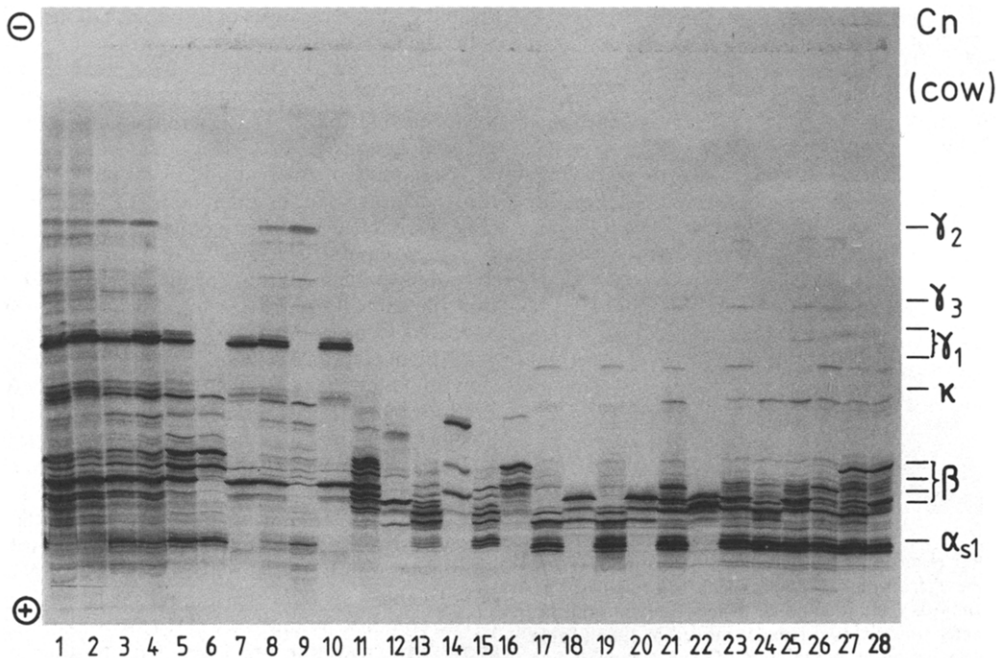


Fig. 1. Thin-layer IEF of Galapagos fur seal milk in comparison to those of other species and to marker proteins. M = whole milk, C = casein fraction, W = whey fraction. First number = female number; last number = pup age (days). Cn = casein. Nos 21-26 bovine casein markers. Position 1: 269, M, 1; 2: 271, M, 1; 3: 269, M, 4; 4: 271, M, 6; 5: 269, M, 12; 6: 269, C, 12; 7: 269, W, 12; 8: 271, M, 12; 9: 271, C, 12; 10: 271, W, 12; 11: C, horse; 12: W, horse; 13: C, goat; 14: W, goat; 15: C, sheep; 16: W, sheep; 17, 19: C, water buffalo; 18, 20: W, water buffalo; 21: C, cow; 22: W, cow; 23: C-marker with  $\beta$ -Cn A2/A1; 24: C-marker with  $\beta$ -Cn A2; 25: C-marker with  $\beta$ -Cn A1; 26: C-marker with  $\beta$ -Cn A2/B; 27: C-marker with  $\beta$ -Cn A2/C; 28: C-marker with  $\beta$ -Cn A1/C.

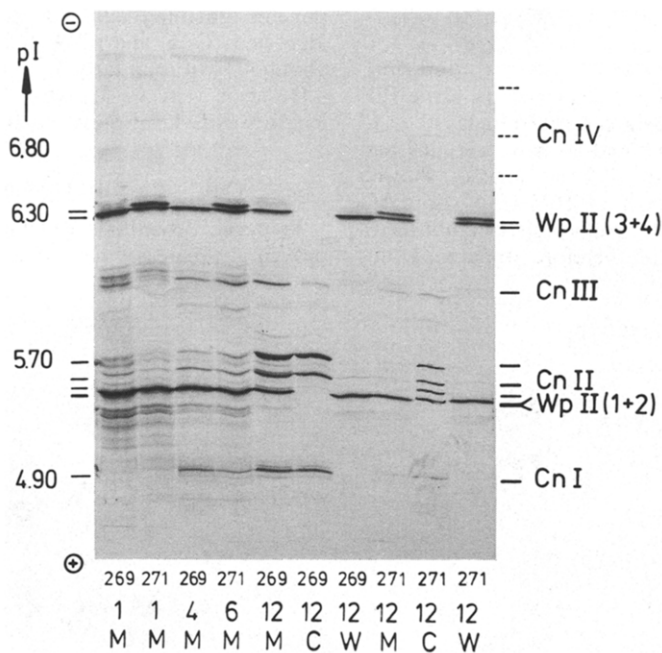


Fig. 2. Thin-layer IEF of whole milks, caseins and whey proteins from Galapagos fur seal females 269 and 271. Symbols; upper line, female number; middle line, age of pup in days; lower line, abbreviations as in Fig. 1.

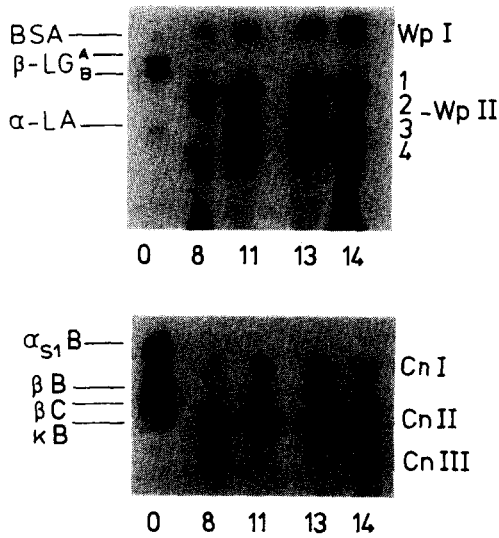


Fig. 3. CAG electrophoresis of whey proteins (above) and caseins (below) from Galapagos fur seals in comparison to bovine standards (0) with known genetic polymorphisms. Numbers refer to individual females milked in 1983 (Table 1). BSA = bovine serum albumin.  $\beta$ -LG =  $\beta$ -lactoglobulins A and B.  $\alpha$ -LA =  $\alpha$ -lactalbumin.

*Whey proteins*

Wp-I (similar to bovine serum albumin = BSA) had a high mobility and could only be resolved in CAG electrophoresis. The *pI*s of proteins in the major whey protein fraction Wp-II were 5.5 (Wp-II, 1), 6.29 (Wp-II, 3) and 6.31 (Wp-II, 4) (Fig. 2). The more basic fraction corresponded to the slowest moving component in CAG electrophoresis. In CAG electrophoresis, Wp-II consisted of up to four well-separated protein zones (Figs 3–5).

*Protein polymorphisms*

Individual females always displayed the same IEF and electrophoretic protein pattern from days 1 to 12 or 15 of lactation (Figs 2 and 5 for females 269 and 271). However, milk of *different* females showed variable banding patterns in IEF (Fig. 2), CAG (Figs 3–5) and PAG electrophoresis (unpublished). The variants found are therefore apparently not

related to different stages of lactation, but more likely reflect genetic polymorphisms.

Within the Wp-II group, the four clearly separated bands (Figs 3–5) suggest a two locus system with two alleles each (Table 3). Wp-I (serum albumin) seems to be homogenous.

The caseins of fur seal milk were more similar to those of other species than were the whey proteins (Fig. 1). Thus a tentative nomenclature of  $\alpha$ -s-,  $\beta$ -, and  $\kappa$ -caseins is suggested. CAG electrophoresis indicated polymorphism forms in the Cn-I fraction (presumed  $\alpha$ -s-casein) (Fig. 4). Female 272 appeared to be homozygous for a more acidic form with higher mobility (Cn-I, 1) while female 273 showed a more basic band with lower mobility (Cn-I, 2). Both bands were present in animals 269 and 271. Stronger banding of the homozygous state in females 272 and 273 agrees with the expected dose effect. These polymorphic forms were not separated in IEF and had a single band at *pI* 4.9; however, the IEF system used also does not separate bovine  $\alpha$ -s-casein variants B and C which have close *pI*-values of 4.92 and 4.95 (Krause, unpublished).

The complex pattern of Cn-II ( $\beta$ -Cn-like) did not allow any clear interpretation. One to four bands were recognizable in CAG electrophoresis (Fig. 4) although separation was not as clear as for the whey proteins. In IEF, milk of female 269 had only two bands of Cn-II (*pI* 5.6 and 5.7) whereas the milk of female 271 showed a further two, which were more acidic components with *pI*s of 5.5 and 5.55 (Fig. 2). Cn-III ( $\kappa$ -Cn) seemed to be homogenous.

*Fatty acid composition*

Three GLC runs were averaged to give the values in Table 4. Only area per cent are given as the identity of a few peaks out of the 22 could not be ascertained due to the lack of standards. Eight minor components, totalling 5.6% of peak areas, were not identified. C-16 and C-18 fatty acids contributed about 70% of total fatty acids and 63% of the total fatty acids were unsaturated. The minimum chain length was C-12 and the maximum C-22.

DISCUSSION

Preparative methods developed for bovine milk proved adequate for the separation of fur seal whey

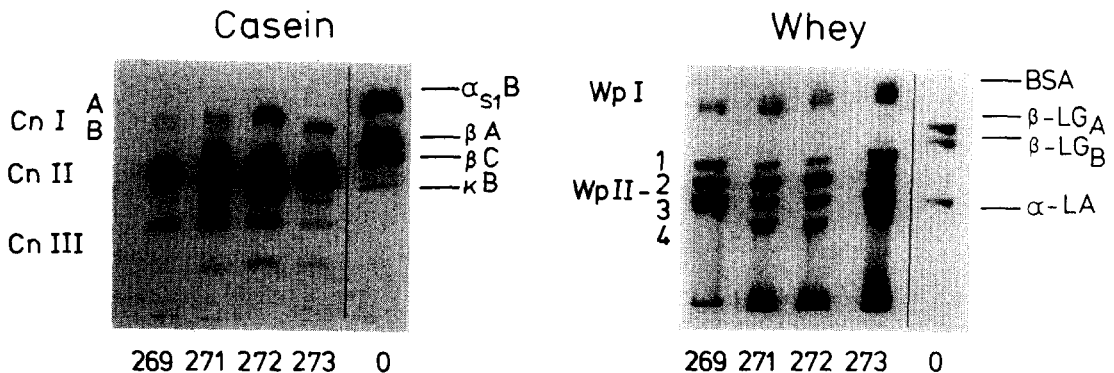


Fig. 4. Comparison of the CAG banding pattern of caseins and whey proteins of the females milked in 1984. 0 = bovine standard, other abbreviations as in Fig. 3.

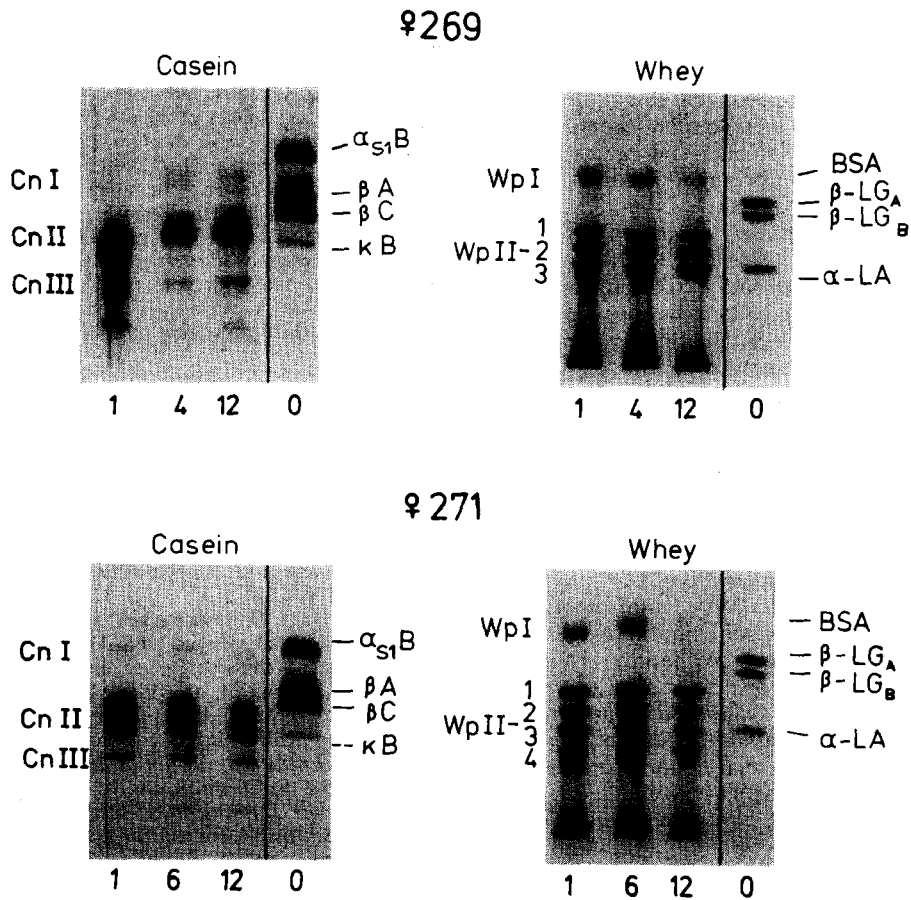


Fig. 5. CAG electrophoresis of caseins and whey proteins of the milk of females 269 and 271. At various stages of lactation banding patterns are always the same. 0 = bovine standard; other numbers = age of pup. Other abbreviations as in Fig. 3.

and casein. The pattern of change in the ratio of casein to whey protein in initial lactation corresponds to that found in many other mammals where, initially, relatively more whey proteins are produced, presumably to transfer immunoglobulins to the young (Cavagnolo and Vedros, 1979; Oftedal, 1984). Caseins became more predominant later in lactation. The 1.6:1 ratio between casein and whey in the Galapagos fur seal milk roughly agrees with data on phocid milks (Jenness and Sloan, 1970) and Northern fur seal milk (Ashworth *et al.*, 1966), but the 3:1 ratio on day 150 is clearly higher. The NPN-values in Table 2 may be slight overestimates since the samples were preserved with Na-azide, initially at room temperature, and so milk proteases may have degraded proteins a little.

Table 3. Whey protein variants in Wp-II, in order of diminishing mobility, as found in the milk of eight fur seals

Component	Female number							
	8	11	13	14	269	271	272	273
Whey	1	+	+	+	+	+	+	+
Protein	2	+	+	+	-	+	+	+
Variants	3	-	+	+	+	+	+	+
	4	+	+	+	+	-	+	-

Table 4. Fatty acid composition of milk fat from Galapagos fur seals. Average of three GLC-runs (in area per cent). x = unidentified fatty acid

Peak no.	Retention time (min)	Fatty acid	Area per cent
1	12.43	x	0.27
2	13.47	x	0.27
3	14.57	C14:0	3.18
4	15.62	x	0.61
5	16.72	C16:0	20.75
6	16.93	C16:1	5.65
7	17.53	x	0.76
8	17.63	C17:0	0.60
9	17.85	x	1.09
10	18.63	C18:0	2.40
11	18.84	C18:1	40.65
12	19.19	C18:2	1.41
13	19.76	C18:3	0.28
14	20.35	C20:0	0.08
15	20.53	C20:1	2.02
16	21.37	C20:4	1.82
17	21.92	C20:5	1.97
18	22.17	C22:2	0.10
19	23.09	x	0.45
20	23.37	x	0.57
21	23.76	x(C22:5?)	1.57
22	24.10	C22:6	6.12

Even tentative homologization of fur seal milk whey components with those of bovine milk seems premature, except perhaps for blood serum albumin. Alpha-lactalbumin appeared to be absent, which is as expected since fur seal milk does not contain lactose (Schmidt *et al.*, 1971; Trillmich and Lechner, 1986). Fur seal caseins were surprisingly similar to those of artiodactyls (Fig. 1). Nevertheless the homology remains speculative until sequencing proves genetic homology (Eigel *et al.*, 1984).

The variants of whey and casein proteins in the electrophoretic banding patterns were apparently not related to the stage of lactation. Such polymorphism in milk proteins may be a valuable addition to studies of genetic differences between seal populations since seal blood proteins generally show little genetic variation (Lidicker *et al.*, 1981; Testa, 1986). Similar polymorphisms of whey proteins were previously described by Shaughnessy (1974) for the Southern elephant seal.

The fatty acid composition is similar to that in other pinnipeds where palmitic (16:0), palmitoleic (16:1), and oleic acid (18:1) also predominate (Oftedal *et al.*, 1986). Possible influences of dietary lipids on milk fatty acid composition have been discussed previously (Ashworth *et al.*, 1966; Stull *et al.*, 1967; van Horn and Baker, 1971), but cannot be substantiated for the Galapagos fur seal as we lack data on fatty acids in their food organisms. The broad pattern of long chain, unsaturated fatty acids seems to be typical for carnivora in general (Glass *et al.*, 1967).

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