

ORIGINAL ARTICLE

Chemical characterization of milk oligosaccharides of an African lion (*Panthera leo*) and a clouded leopard (*Neofelis nebulosa*)

Akitsugu SENDA,¹ Emi HATAKEYAMA,¹ Rui KOBAYASHI,² Kenji FUKUDA,¹ Yusuke UEMURA,³ Tadao SAITO,⁴ Craig PACKER,⁵ Olav T. OFTEDAL⁶ and Tadasu URASHIMA¹

¹Graduate School of Food Hygiene and ²Department of Bioresource Chemistry, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, ³Department of Chemistry, School of Science, Kitasato University, Sagami-hara, ⁴Graduate School of Agriculture, Tohoku University, Aoba, Sendai, Japan, ⁵Department of Ecology, Evolution and Behavior, University of Minnesota, St. Paul, MN, and ⁶Smithsonian Environmental Research Center, Edgewater, MD, USA

ABSTRACT

The Carnivora include the superfamilies Canoidea and Feloidea. In species of Canoidea other than the domestic dog, *Canis lupus*, the milk contains only traces of lactose and much larger concentrations of oligosaccharides. In this study, lactose was found to be the dominant saccharide in the milk or colostrum of two species of Feloidea, namely the African lion (*Panthera leo*) and the clouded leopard (*Neofelis nebulosa*). In addition to lactose, the following oligosaccharides were characterized in the milk of a lion; Neu5Gc(α2-3)Gal(β1-4)Glc (3'-NGc-SL), Fuc(α1-2)Gal(β1-4)Glc (2'-fucosyllactose) and GalNAc(α1-3)[Fuc(α1-2)]Gal(β1-4)Glc (A-tetrasaccharide). The colostrum of a clouded leopard contained 3'-NGc-SL, Gal(α1-3)Gal(β1-4)Glc (isoglobotriose) and A-tetrasaccharide. These oligosaccharides differ in some respects from those previously identified in another species of Feloidea, the spotted hyena (*Crocuta crocuta*). These milks contained 3'-NGc-SL and A-tetrasaccharide, while spotted hyena colostrum did not; however, it contained Neu5Ac(α2-3)Gal(β1-4)Glc (3'-Nac-SL) and Gal(α1-3)[Fuc(α1-2)]Gal(β1-4)Glc (B-tetrasaccharide).

Key words: African lion, carnivora, clouded leopard, felidae, milk oligosaccharides.

INTRODUCTION

The milk of eutherian mammals usually contains lactose as the dominant saccharide (Jenness *et al.* 1964) in addition to low concentrations of a variety of oligosaccharides, almost all of which contain a lactose unit at their reducing ends. However, milk oligosaccharides predominate over lactose in the milks of the white-nosed coati (*Nasua narica*, Procyonidae) (Urashima *et al.* 1999a), mink (*Mustela vison*, Mustelidae) (Urashima *et al.* 2005), brown bear (*Ursus arctos*, Ursidae) (Urashima *et al.* 1997a), Japanese black bear (*Ursus thibetanus japonicus*, Ursidae) (Urashima *et al.* 1999b, 2004a), polar bear (*Ursus maritima*, Ursidae) (Urashima *et al.* 2000, 2003a), giant panda (*Ailuropoda melanoleuca*, Ursidae) (Nakamura *et al.* 2003), hooded seal (*Cystophora cristata*, Phocidae) (Urashima *et al.* 2001), harbour seal (*Phoca vitulina*, Phocidae) (Urashima *et al.* 2003b) and bearded seal (*Erignathus barbatus*, Phocidae) (Urashima *et al.* 2004b). These species all belong to the superfamily Canoidea of the

order Carnivora, a taxon encompassing a variety of omnivores, generalized carnivores and marine carnivores, such as dogs and foxes (Canidae), bears (Ursidae), raccoons (Procyonidae), weasels (Mustelidae) and the pinniped families (Otariidae, Phocidae, Odobenidae). These observations suggest that Canoidea may differ from other studied eutherians in having a low ratio of lactose to milk oligosaccharides in their milk and colostrum. However, in milk of the domestic dog lactose reportedly comprises approximately 90% of the carbohydrate fraction (Bubb *et al.* 1999). The milks of species of Canoidea are characteristic, insofar as many of them contain oligosaccharides

Correspondence: Tadasu Urashima, Graduate school of Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan. (Email: urashima@obihiro.ac.jp)

Received 7 July 2009; accepted for publication 12 January 2010.

that include A (GalNAc(α 1-3)[Fuc(α 1-2)]Gal), B (Gal(α 1-3)[Fuc(α 1-2)]Gal), H (Fuc(α 1-2)Gal) or α -Gal epitope (Gal(α 1-3)Gal) at the non-reducing ends.

In contrast to the Canioidea, little work has been done on milk composition of the Felioidea, a superfamily including such specialized carnivores as the civets and mongooses (Viverridae), hyenas (Hyaenidae) and cats (Felidae) (Oftedal & Gittleman 1989; Oftedal & Iverson 1995). Oligosaccharides have been reported from a sample of colostrum of the spotted hyena (*Crocuta crocuta*, Hyaenidae), which was found to contain lactose as the dominant saccharide in addition to small amounts of Neu5Ac(α 2-3)Gal(β 1-4)Glc, Gal(α 1-3)[Fuc(α 1-2)]Gal(β 1-4)Glc, Gal(α 1-3)Gal(β 1-4)Glc and Fuc(α 1-2)Gal(β 1-4)Glc (Uemura *et al.* 2009). Osthoff *et al.* (2006) reported that cheetah (*Acinonyx jubatus*, Felidae) milk contains 40.2 g/kg lactose and 0.22 g/kg milk oligosaccharides, suggesting that the milk/colostrum of Felioidea may differ from those of most Canioidea with respect to the ratio of milk oligosaccharides to lactose. In this study, we have characterized the milk oligosaccharides of two species of Felioidea, the African lion (*Panthera leo*, Felidae) and the clouded leopard (*Neofelis nebulosa*, Felidae), and report on the characteristic differences with respect to milk oligosaccharides between these two species and the spotted hyena.

MATERIALS AND METHODS

Materials

The African lion (*Panthera leo*) milk sample (45 mL) was collected on August 6, 1986 from a lactating female (individual ID: CSN) in Serengeti National Park, Tanzania at 127 days postpartum. The female was anesthetized with telazol and injected 1 mol/L with oxytocin prior to manual expression of milk. This lion population has been the subject of extensive field research, including detailed studies of nursing behavior and maternal care (e.g. Pusey & Packer 1994). The clouded leopard (*Neofelis nebulosa*) colostrum samples (2 and 6 mL) were manually collected one day postpartum from a lactating clouded leopard at the Seneca Park Zoo (Rochester NY, USA) on July 9, 1986 during a medical examination. The two clouded leopard samples were from different nipples, but were combined for use in this study. The samples were stored at -20°C until use.

Fuc(α 1-2)Gal(β 1-4)Glc (2'-fucosyllactose) and GalNAc(α 1-3)[Fuc(α 1-2)]Gal(β 1-4)Glc (A-tetrasaccharide) were purchased from Sigma (St. Louis, MO, USA) and Funakoshi (Tokyo, Japan), respectively. Neu5Gc(α 2-3)Gal(β 1-4)Glc (3'-NGc-Lac) and Gal(α 1-3)Gal(β 1-4)Glc (isoglobotriose) were purified from ovine colostrum (Nakamura *et al.* 1998) and caprine colostrum (Urashima *et al.* 1994), respectively.

Isolation of milk oligosaccharides and lactose

The milk/colostrum samples were thawed and extracted with four volumes of chloroform/methanol (2:1, v/v).

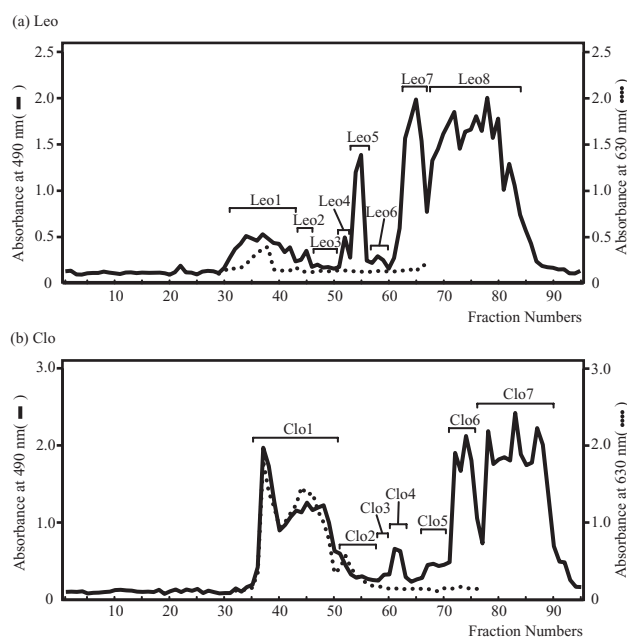


Figure 1 Gel chromatograms of the crude carbohydrate fractions extracted from (a) lion milk and (b) clouded leopard colostrum using a Bio Gel P-2 extra fine column (2.6×100 cm). Fractions were monitored by the phenol- H_2SO_4 method (OD_{490}) and the periodate-resorcinol method (OD_{630}).

The emulsions were centrifuged at 4°C and $4000 \times g$ for 30 min, and the lower chloroform layer and the denatured proteins were discarded. The methanol was removed from the upper layer by rotary evaporation, and the residues were dissolved in 30 mL water and freeze-dried. The resulting white powders were called the 'carbohydrate fractions'.

The carbohydrate fractions were each dissolved in 2 mL of water and the solutions passed through a Bio Gel P-2 ($<45 \mu\text{m}$, Bio-Rad Laboratories, Hercules, CA, USA) column (2.5×100 cm) that had been calibrated with 2 mg each of galactose (monosaccharide), lactose (disaccharide) and raffinose (trisaccharide). Elutions were done with distilled water at a flow rate of 15 mL/h and fractions of 5 mL were collected. Aliquots (0.5 mL) of each fraction were analyzed for hexose with phenol- H_2SO_4 (Dubois *et al.* 1956) and for sialic acid with periodate-resorcinol (Jourdain *et al.* 1971). Peak fractions were pooled and freeze-dried. The saccharides in the peak fractions Leo-8, Leo-7, Leo-5, Clo-7, Clo-6 and Clo-4 were subjected to proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy to determine their chemical structures.

The components of peaks Leo-1 (see Fig. 1a) and Clo-1 (see Fig. 1b), which gave positive reactions with both periodate-resorcinol (630 nm) and phenol- H_2SO_4 (490 nm), were each dissolved in 2 mL of 50 mmol/L Tris hydroxymethylmethane-HCl buffer (pH 8.7) and subjected to anion exchange chromatography on diethylaminoethyl (DEAE)-Sephadex A-50 (GE Healthcare, Uppsala, Sweden). The unadsorbed components were eluted with 250 mL of the same buffer and the adsorbed components were then eluted with a linear gradient of 0–0.5 mol/L NaCl in the Tris buffer solution. Elution was done at a flow rate of 15 mL/h and

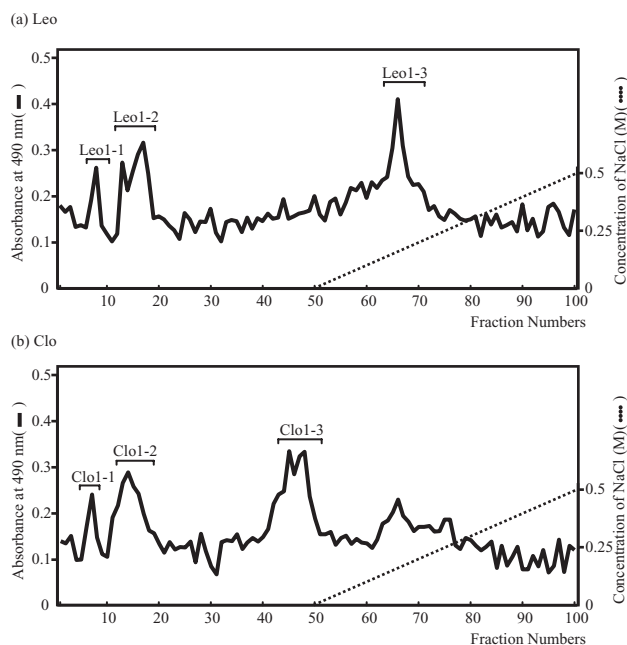


Figure 2 Anion exchange chromatograms of (a) fraction Leo-1 and (b) fraction Clo-1 separated in Fig. 1a and b, respectively, using a diethylaminoethyl (DEAE)-Sephadex A-50 column (1.5 × 40 cm). Fractions were monitored by the phenol-H₂SO₄ method (OD₄₉₀).

fractions of 5 mL were collected. Aliquots (0.5 mL) of each fraction were analyzed for hexose using the phenol-H₂SO₄ method. The fractions in Leo-1-2 and Clo-1-2 (see Fig. 2a,b) were pooled, lyophilized, dissolved in 2 mL of water and passed through a column with Bio Gel P-2 as described above, to remove salt.

The components in Leo-1-2 and Clo-1-2 (see Fig. 2a,b) were further separated by high-performance liquid chromatography (HPLC) on a TSK gel Amido 80 column (4.6 × 250 mm, pore size 80 Å, particle size 5 μm; Tosoh, Tokyo, Japan) using a LC-10ATVP pump (Shimadzu, Tokyo, Japan) (chromatogram on Fig. 3a,b). The mobile phase was 50% and 80% (v/v) acetonitrile (CH₃CN) in 15 mmol/L potassium phosphate buffer (pH 5.2). Elution was done using a linear gradient of acetonitrile from 80% to 50% at 60°C at a flow rate of 1 mL/min. The eluates were monitored by measuring the absorbance at 195 nm. The peak fractions of oligosaccharides were pooled, concentrated by rotary evaporation, and subjected to ¹H-NMR spectroscopy to determine the structures.

¹H-NMR spectroscopy

Nuclear magnetic resonance spectra were recorded in D₂O (100.00 atom D%; Aldrich, Milwaukee, WI, USA) at 500 or 600 MHz for ¹H-NMR with a JEOL ECP-500 FT-NMR (Akishima, Tokyo, Japan) or a Varian INOVA 600 spectrometer (Palo Alto, CA, USA) operated at 293.1 K. Chemical shifts are expressed in δ relative to internal 3-(trimethylsilyl)-1-propane sulfonic acid, sodium salt (TPS), but actually measured by reference to internal acetone (δ = 2.225).

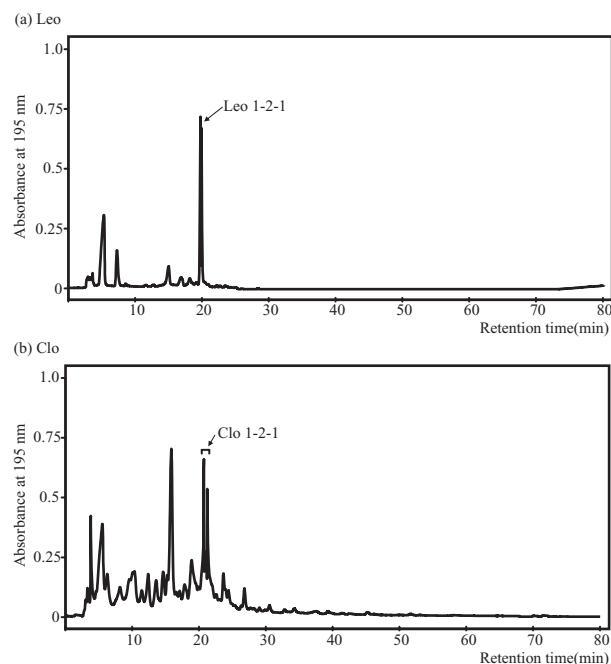


Figure 3 High-performance liquid chromatography (HPLC) profiles of the acidic oligosaccharide fraction (a) Leo-1-2 and (b) Clo-1-2, separated from lion milk and clouded leopard colostrums, respectively. HPLC was done using a Shimadzu LC-10ATVP pump on a TSK gel Amido 80 column (4.6 × 250 mm, pore size = 80 Å, particle size = 5 μm). The mobile phase was 50% and 80% acetonitrile in 15 mmol/L potassium phosphate buffer. Elution was done using a linear gradient of acetonitrile from 80% to 50% at 60°C at a flow rate of 1 mL/min. The detection of peaks was done by UV absorbance at 195 nm.

RESULTS

The carbohydrate fractions of the lion milk and clouded leopard colostrum were resolved into several peaks by chromatography on Bio Gel P-2 as shown in Figure 1a,b. The components in each fraction were pooled and designated as in Figure 1a,b. The saccharides in Leo-8, Leo-7, Leo-5 from the lion milk, and Clo-4, Clo-6 and Clo-7 from the clouded leopard colostrum were characterized by ¹H-NMR.

Lion neutral oligosaccharides

Leo-8

As the ¹H-NMR spectrum of the saccharide in Leo-8 (chemical shifts in Table 1) was completely identical with that of lactose, it was characterized as Gal(β1-4)Glc.

Leo-7

As the ¹H-NMR spectrum of Leo-7 (chemical shifts in Table 1) was completely identical with that of authentic 2'-FL, the saccharide in this fraction was identified as Fuc(α1-2)Gal(β1-4)Glc.

Table 1 Proton nuclear magnetic resonance (¹H-NMR) chemical shifts of the oligosaccharides separated from African lion milk

Reporter group	Residue	Chemical shifts, δ (coupling constants, Hz)			
		Leo1-2-1	Leo5	Leo7	Leo8
H-1	Glc α	5.221 (2.3)	5.227 (3.4)	5.225 (8.0)	5.222 (3.4)
	Glc β	4.665 (8.0)	4.631 (8.0)	4.638 (4.0)	4.664 (8.0)
	Gal β 4	4.534 (8.0)	4.585 (7.4)	4.527 (8.0)	4.450 (8.0)
	Fuc α 2	–	5.351 (4.0)	5.312 (2.3)	–
	GalNAc α 3	–	5.177 (4.0)	–	–
H-2	GalNAc α 3	–	4.225 (3.4), 4.248 (4.0)	–	–
H-3	Gal β 4	4.139	–	–	–
H-3 _{ax}	Neu5Ac(α 2-3)	1.809 (12.0, -12.0)	–	–	–
H-3 _{eq}	Neu5Ac(α 2-3)	2.776 (4.5)	–	–	–
H-4	Gal β 4	–	4.225 (3.4)	–	–
H-5	GalNAc α 3	–	4.225 (3.4)	–	–
	Fuc α 2	–	4.325, 4.369	4.259, 4.230	–
H-6	Fuc α 2	–	1.244 (6.3)	1.225 (6.9)	–
NAC	GalNAc α 3	–	2.041	–	–
NGc	Neu5Gc(α 2-3)	4.119	–	–	–

Leo1-2-1, Neu5Gc(α 2-3)Gal(β 1-4)Glc; Leo5, GalNAc(α 1-3)[Fuc(α 1-2)]Gal(β 1-4)Glc; Leo7, Fuc (α 1-2)Gal(β 1-4)Glc; Leo8, Gal(β 1-4)Glc.

The spectrum had the anomeric signals of α (1-2) linked Fuc, α -Glc, β -Glc and β (1-4) linked Gal at δ 5.312, 5.225, 4.638 and 4.527, respectively, and H-5 and H-6 of α (1-2) linked Fuc at δ 4.259, 4.230 and 1.225, respectively.

Leo-5

As the ¹H-NMR spectrum (chemical shifts in Table 1) was completely identical with that of authentic A-tetrasaccharide, the saccharide in this fraction was identified as GalNAc(α 1-3)[Fuc(α 1-2)]Gal(β 1-4)Glc.

The spectrum had the anomeric signals of α (1-2) linked Fuc, α (1-3) linked GalNAc, α -Glc, β -Glc and β (1-4) linked Gal at δ 5.351, 5.227, 5.177, 4.631 and 4.585, respectively. It contained the characteristic signals of H-5 and H-6 of α (1-2) linked Fuc at δ 4.369, 4.325 and 1.244, respectively, H-2 of α (1-3) linked GalNAc (α) at δ 4.248, H-4 of β (1-4) linked Gal, which was substituted by α (1-3) linked Gal, at δ 4.225, and H-5 of α (1-3) linked GalNAc at δ 4.225.

Lion acidic oligosaccharides

The components in Leo-1, which contained sialic acid, were subjected to anion exchange chromatography. The unadsorbed fractions were designated as Leo-1-1 and Leo-1-2 as shown in Figure 2a. The crude sialyl oligosaccharide fraction, Leo-1-2, was passed through a Bio Gel P-2 column and each oligosaccharide was separated by HPLC (chromatogram in Fig. 3a) prior to characterization by ¹H-NMR. The components in Leo-1-1, which were assumed to be higher neutral oligosaccharides, were not characterized in this study, because the content of this fraction was too small to be characterized. The oligosaccharide in Leo-1-2-2 (Fig. 3a), which was purified by HPLC, was characterized by ¹H-NMR.

Leo-1-2-1

As the ¹H-NMR spectrum (chemical shifts in Table 1) of Leo-1-2-2 had a NGc signal at δ 4.119 but no NAC signal, it was speculated that the saccharide in this fraction contained N-glycolylneuraminic acid. The existence of H-3 axial and equatorial signals of sialic acid at δ 1.809 and 2.776 showed that the saccharide had an Neu5Gc(α 2-3)Gal unit. The spectrum had the anomeric signals of α -Glc, β -Glc and β (1-4) linked Gal at δ 5.221, 4.665 and 4.534, respectively, and the characteristic H-3 signal of β (1-4) linked Gal at δ 4.139. As this pattern was essentially similar to that of authentic 3'-N-glycolylneuraminylactose, the saccharide in Leo-1-2-2 was identified as Neu5Gc(α 2-3)Gal(β 1-4)Glc.

Clouded leopard neutral oligosaccharides

Clo-7

As the ¹H-NMR spectrum of Clo-7 (chemical shifts in Table 2) was identical to that of lactose, the saccharide in Clo-7 was identified as Gal(β 1-4)Glc.

Clo-6

As the ¹H-NMR spectrum of Clo-6 (chemical shifts in Table 2) was essentially similar to that of authentic isoglobotriose, the saccharide in Clo-6 was identified as Gal(α 1-3)Gal(β 1-4)Glc. The spectrum had the anomeric signals of α -Glc, α (1-3) linked Gal, β -Glc and β (1-4) linked Gal at δ 5.226, 5.146, 4.669 and 4.524, respectively, H-5 of α (1-3) linked Gal at δ 4.196, and H-4 of β (1-4) linked Gal, which was substituted by α (1-3) linked Gal, at δ 4.184.

Clo-4

As the ¹H-NMR spectrum of Clo-4 (chemical shifts in Table 2) was essentially similar to that of authentic

Table 2 Proton nuclear magnetic resonance (¹H-NMR) chemical shifts of the oligosaccharides from clouded leopard colostrum

Reporter group	Residue	Chemical shifts, δ (coupling constants, Hz)			
		Clo1-2	Clo4	Clo6	Clo7
H-1	Glc α	5.220 (4.1)	5.228 (3.4)	5.226 (3.4)	5.223 (4.0)
	Glc β	4.663 (8.0)	4.632 (7.4)	4.669 (8.0)	4.665 (8.0)
	Gal β 4	4.534 (8.0)	4.586 (7.4)	4.524 (7.4)	4.450 (8.0)
	Fuc α 2	–	5.352 (4.0)	–	–
	GalNAc α 3	–	5.176 (4.0)	–	–
H-2	Gal α 3	–	–	5.146 (4.0)	–
	GalNAc α 3	–	4.225 (2.9), 4.249 (4.0)	–	–
H-3	Gal β 4	4.135 (2.9)	–	–	–
H-3 _{ax}	Neu5Gc(α 2-3)	1.816 (12.0, -12.0)	–	–	–
H-3 _{eq}	Neu5Gc(α 2-3)	2.775 (4.5)	–	–	–
H-4	Gal β 4	–	4.225 (2.9)	4.184 (2.9)	–
H-5	Gal α 3	–	–	4.196	–
	GalNAc α 3	–	4.225 (2.9)	–	–
	Fuc α 2	–	4.325, 4.370	–	–
H-6	Fuc α 2	–	1.248 (6.3)	–	–
NAc	GalNAc α 3	–	2.042	–	–
NGc	Neu5Gc(α 2-3)	4.115	–	–	–

Clo1-2, Neu5Gc(α 2-3)Gal(β 1-4)Glc; Clo 4, GalNAc(α 1-3)[Fuc(α 1-2)]Gal(β 1-4)Glc; Clo 6, Gal(α 1-3)Gal(β 1-4)Glc; Clo 7, Gal(β 1-4)Glc.

A-tetrasaccharide and also of Leo-5, the saccharide in Clo-4 was identified as GalNAc(α 1-3)[Fuc(α 1-2)]Gal(β 1-4)Glc.

Clouded Leopard acidic oligosaccharides

The components in Clo-1, which contained sialic acid, were subjected to anion exchange chromatography. The unadsorbed fractions were designated as Clo-1-1 and Clo-1-2 as shown in Fig. 2b. The crude sialyl oligosaccharide fraction, Clo-1-2, was passed through a Bio Gel P-2 column and each oligosaccharide was separated by HPLC (chromatogram in Fig. 3b) prior to characterization by ¹H-NMR. The components in Clo-1-1, which were assumed to be higher neutral oligosaccharides, were not characterized in this study, because the content of this fraction was too small. The oligosaccharide in Clo-1-2 (see Fig. 3b), which was purified by HPLC, was characterized by ¹H-NMR.

Clo-1-2

As the ¹H-NMR of Clo-1-2 was essentially similar to that of authentic 3'-N-glycolylneuraminylactose and also of Leo-1-2-2, the saccharide in Clo-1-2 was identified as Neu5Gc(α 2-3)Gal(β 1-4)Glc.

DISCUSSION

In the colostrum of clouded leopard, the ratio of milk oligosaccharides to lactose was estimated by comparison of the peak areas in the Bio Gel P-2 column chromatography in Figure 1a to be 1:1, while in the milk of the African lion, it was estimated to be 1.0:2.3 (Fig. 1b). The value for clouded leopard was similar to that for spotted hyena (Uemura *et al.* 2009), while that for the lion was lower. It is likely that this difference was due to the differences between the lactation

periods at which these samples were collected, namely colostrum versus mature milk. It is clear that lactose is the dominant carbohydrate in the milk or colostrum of all three Felidae species; this situation differs from that of the Canidae other than the dog. Although it is still unknown whether lactose is a prominent saccharide in the milks of all Felidae, the predominance of lactose in the milk of the above three species suggests that this disaccharide is physiologically significant for their neonates in contrast to those of Canidae other than dog. As is well known, lactose is usually an important energy source for mammalian neonates. Our results show that this is true also for the neonates of these three species. On the other hand, we have no explanation for the decrease in the ratio of lactose in the milk carbohydrate of Canidae other than dog.

The milk carbohydrates of the African lion, clouded leopard and spotted hyena, which were identified in this study and in our previous study (Uemura *et al.* 2009), are shown in Table 3. The milk oligosaccharides found in lion milk and clouded leopard colostrum were similar insofar as 3'-NGc-Lac and A-tetrasaccharide were detected in both. On the other hand, these oligosaccharides were not detected in spotted hyena colostrum, but 3'-N-acetylneuraminylactose (3'-NAC-Lac) and B-tetrasaccharide were found. However, one should note that the milk oligosaccharides are rather similar in these species in that their milk or colostrum was found to contain 3'-sialyllactose but not 6'-sialyllactose and all contained the oligosaccharides containing A (GalNAc(α 1-3)[Fuc(α 1-2)]Gal) or B (Gal(α 1-3)[Fuc(α 1-2)]Gal) antigen. A-tetrasaccharide has also been found in the milk of bonobo (Urashima *et al.* 2009), minke whale (Urashima *et al.* 2002) and polar bear (Urashima *et al.* 2000), whereas 3'-NGc-Lac

Table 3 Milk oligosaccharides of African lion, clouded leopard and spotted hyena

	African lion	Clouded leopard	Spotted hyena
Neu5Gc(α 2-3)Gal(β 1-4)Glc	•	•	
Neu5Ac(α 2-3)Gal(β 1-4)Glc			•
GalNAc(α 1-3)[Fuc(α 1-2)]Gal(β 1-4)Glc	•	•	
Gal(α 1-3)[Fuc(α 1-2)]Gal(β 1-4)Glc			•
Fuc(α 1-2)Gal(β 1-4)Glc	•		•
Gal(α 1-3)Gal(β 1-4)Glc		•	•
Gal(β 1-4)Glc	•	•	•

has also been found in milk or colostrum of the chimpanzee (Urashima *et al.* 2009), bonobo (Urashima *et al.* 2009), gorilla (Urashima *et al.* 2009), orangutan (Urashima *et al.* 2009) and sheep (Nakamura *et al.* 1998).

Oligosaccharides containing A antigen (GalNAc(α 1-3)[Fuc(α 1-2)]Gal) have been found in the milk or colostrum of bonobo (Urashima *et al.* 2009), minke whale (Urashima *et al.* 2002) and polar bear (Urashima *et al.* 2000), while oligosaccharides containing B antigen (Gal(α 1-3)[Fuc(α 1-2)]Gal) have been found in the milk or colostrum of gorilla (Urashima *et al.* 2009), Japanese black bear (Urashima *et al.* 1999b) and polar bear (Urashima *et al.* 2000). Differences with respect to milk oligosaccharides containing either A or B antigen have been previously observed between closely related species such as bonobo and gorilla, or polar bear and Japanese black bear. It is noteworthy that this difference was also observed between spotted hyena and the closely related above-mentioned two species of Felidae.

Humans are the only eutherian species in which body tissues and fluids lack glycoconjugates containing N-glycolylneuraminic acid (Brinkman-Van der Linden *et al.* 2000). It is believed that this is due to the absence of CMP-Neu5Ac hydroxylase, which converts CMP-Neu5Ac to CMP-Neu5Gc. On the other hand, acidic milk oligosaccharides that contain N-glycolylneuraminic acid have been found in the milk or colostrum of several closely related species including chimpanzee, bonobo, gorilla and orangutan (Urashima *et al.* 2009), as well as in goat (Urashima *et al.* 1997b), sheep (Nakamura *et al.* 1998) and cow (Tao *et al.* 2008). The milk of many species studied to-date contains acidic oligosaccharides containing only N-acetylneuraminic acid (Urashima *et al.* 2007), but ovine colostrum exceptionally contains more N-glycolylneuraminic acid than N-acetylneuraminic acid (Nakamura *et al.* 1998). The African lion and clouded leopard may be unique among the Carnivora insofar as these are the only species so far investigated whose milk or colostrum contain N-glycolylneuraminic acid. As the acidic oligosaccharides of the milk or colostrum of all Canioidea species so far investigated, as well as of the colostrum of a spotted hyena, contain only N-acetylneuraminic acid (Urashima *et al.* 2007; Uemura *et al.* 2009), this situa-

tion may be a specific feature of Feloidae and closely related species. However, additional species should be studied to clarify whether this is applicable to other Feloidae.

It appears that the patterns of milk oligosaccharides of Feloidae species are relatively simple compared with those of most Canioidea species. Canioidea milks have been shown to contain higher oligosaccharides the core units of which are lacto-N-neotetraose, lacto-N-neohexaose or para lacto-N-neohexaose (Urashima *et al.* 2007), whereas the Feloidae milk/colostrum do not contain these oligosaccharides or contain smaller amounts. It can be hypothesized that this is due to absence or low activity of β 3N-acetylglucosaminyltransferase, that is the enzyme, which elongates the milk oligosaccharide chain, in the lactating mammary glands of these three species.

The biological significance of this apparent taxonomic difference between Canioidea and Feloidae is not clear. It has been proposed that a complexity of milk oligosaccharides provides some protection against colonization of the digestive tract by pathogens, which may be particularly important in species with altricial young (such as bears) or species that are highly social (such as humans) (Urashima *et al.* 2007). The two species of fields that we studied both have relatively altricial young, and lions are highly social even to the point of nursing each others' young (Packer *et al.* 1992, 2001; Pusey & Packer 1994), and thus would be predicted to have high complexity of milk oligosaccharides. The fact that we did not observe unusual complexity of milk oligosaccharides in either of these field species suggests that the evolution of high oligosaccharide milks may be more constrained by phylogeny than previously supposed.

ACKNOWLEDGMENTS

This study was partially supported from a grant from Global COE Program 'Frontier Program for Animal Global Health and Hygiene', Ministry of Education, Culture, Sports, Science, and Technology, Japan. We thank Dr Jeff Wyatt of the Seneca Park Zoo, Rochester, NY for providing the clouded leopard colostrum samples, and Tanzania Wildlife Research Institute for authorization to sample lions in Serengeti National

Park, Tanzania. Michael Jakubasz of the Nutrition Laboratory, National Zoological Park, Washington DC assisted with initial processing of the samples.

REFERENCES

- Brinkman-Van der Linden ECM, Sjöberg ER, Juneja LR, Crocker PR, Varki N, Varki A. 2000. Loss of N-glycolylneuraminic acid in human evolution. Implications for sialic acid recognition by siglecs. *Journal of Biological Chemistry* **275**, 8633–8640.
- Bubb WA, Urashima T, Kohso K, Nakamura T, Arai I, Saito T. 1999. Occurrence of an usual lactose sulfate in dog milk. *Carbohydrate Research* **318**, 123–128.
- Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350–356.
- Jenness R, Regehr E, Sloan RE. 1964. Comparative biochemical studies of milks:II. Dialyzable carbohydrates. *Comparative Biochemistry and Physiology* **13**, 339–352.
- Jourdian GW, Dean L, Roseman S. 1971. The sialic acid XI. A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides. *Journal of Biological Chemistry* **256**, 430–435.
- Nakamura T, Urashima T, Mizukami T, Fukushima M, Arai I, Senshu T, Imazu K, Nakao T, Saito T, Ye Z, Zuo H, Wu K. 2003. Composition and oligosaccharides of a milk sample of the giant panda, *Ailuropoda melanoleuca*. *Comparative Biochemistry and Physiology* **B135**, 439–448.
- Nakamura T, Urashima T, Nakagawa M, Saito T. 1998. Sialyllactose occurs as free lactones in ovine colostrum. *Biochimica et Biophysica Acta* **1381**, 286–292.
- Oftedal OT, Gittleman JG. 1989. Patterns of energy output during reproduction in carnivores. In: Gittleman JG (ed.), *Carnivore Behavior, Ecology and Evolution*, pp. 355–378. Cornell University Press, New York.
- Oftedal OT, Iverson SJ. 1995. Phylogenetic variation in the gross composition of milks. In: Jensen R (ed.), *Handbook of Milk Composition*, pp. 749–789. Academic Press, New York.
- Osthoff G, Hugo A, de Wit A. 2006. The composition of cheetah (*Acinonyx jubatus*) milk. *Comparative Biochemistry and Physiology* **B145**, 265–269.
- Packer C, Lewis S, Pusey AW. 1992. A comparative analysis of non-offspring nursing. *Animal Behaviour* **43**, 265–281.
- Packer C, Pusey AE, Eberly LE. 2001. Egalitarianism in female African lions. *Science* **293**, 690–693.
- Pusey AE, Packer C. 1994. Non-offspring nursing in social carnivores: minimizing the costs. *Behavioral Ecology* **5**, 362–374.
- Tao N, DePeters EJ, Freeman S, German JB, Grimm R, Lebrilla CB. 2008. Bovine milk glycome. *Journal of Dairy Science* **91**, 3868–3778. Doi:10.3168/jds.2008-1305.
- Uemura Y, Takahashi S, Senda A, Fukuda K, Saito T, Oftedal OT, Urashima T. 2009. Chemical characterization of milk oligosaccharides of a spotted hyena (*Crocuta crocuta*). *Comparative Biochemistry and Physiology* **A152**, 158–161.
- Urashima T, Arita M, Yoshida M, Nakamura T, Arai I, Saito T, Arnould JPY, Kovacs KM, Lydersen C. 2001. Chemical characterization of the oligosaccharides in hooded seal (*Cystophora cristata*) and Australian fur seal (*Arctocephalus pusillus doriferus*) milk. *Comparative Biochemistry and Physiology* **B128**, 307–323.
- Urashima T, Asakuma S, Messer M. 2007. Milk oligosaccharides. In: Kamerling J, Boons G-J, Lee YC, Suzuki A, Taniguchi N, Jorjage AGJ (eds), *Comprehensive Glycoscience: From Chemistry to System Biology*, vol. 4. pp. 694–724. Elsevier Science, Amsterdam, Netherlands.
- Urashima T, Bubb WA, Messer M, Tsuji Y, Taneda Y. 1994. Studies of the neutral trisaccharides of goat (*Capra hircus*) colostrum and of the one- and two-dimensional ¹H- and ¹³C NMR spectra of 6'-N-acetylglucosaminylactose. *Carbohydrate Research* **262**, 173–184.
- Urashima T, Kusaka Y, Nakamura T, Saito T, Maeda N, Messer M. 1997a. Chemical characterization of milk oligosaccharides of the brown bear, *Ursus arctos yesoensis*. *Biochimica et Biophysica Acta* **1334**, 247–255.
- Urashima T, Murata S, Nakamura T. 1997b. Structural determination of monosialyl trisaccharides obtained from Caprine colostrum. *Comparative Biochemistry and Physiology* **B116**, 431–435.
- Urashima T, Nagata H, Nakamura T, Arai I, Saito T, Imazu K, Hayashi T, Derocher AE, Wiig O. 2003a. Differences of oligosaccharides pattern of a sample of polar bear colostrum and mid-lactation milk. *Comparative Biochemistry and Physiology* **B136**, 887–896.
- Urashima T, Nakamura T, Ikeda A, Asakuma S, Arai I, Saito T, Oftedal OT. 2005. Characterization of oligosaccharides in milk of a mink, *Mustela vison*. *Comparative Biochemistry and Physiology* **A142**, 461–471.
- Urashima T, Nakamura T, Nakagawa D, Noda M, Arai I, Saito T, Lydersen C, Kovacs KM. 2004b. Characterization of oligosaccharides in milk of bearded seal (*Erignathus barbatus*). *Comparative Biochemistry and Physiology* **B138**, 1–18.
- Urashima T, Nakamura T, Teramoto K, Arai I, Saito T, Komatsu T, Tsubota T. 2004a. Chemical characterization of sialyl oligosaccharides in milk of the Japanese black bear, *Ursus thibetanus japonicus*. *Comparative Biochemistry and Physiology* **B139**, 587–595.
- Urashima T, Nakamura T, Yamaguchi K, Munakata J, Arai I, Saito T, Lydersen C, Kovacs KM. 2003b. Chemical characterization of the oligosaccharides in milk of high Arctic harbour seal (*Phoca vitulina vitulina*). *Comparative Biochemistry and Physiology* **A135**, 549–563.
- Urashima T, Odaka G, Asakuma S, Uemura Y, Goto K, Senda A, Saito T, Fukuda K, Messer M, Oftedal OT. 2009. Chemical characterization of oligosaccharides in chimpanzee, bonobo, gorilla, orangutan and siamang milk or colostrum. *Glycobiology* **19**, 499–508. doi:10.1093/glycob/cwp0006.
- Urashima T, Sato H, Munakata J, Nakamura T, Arai I, Saito T, Tetsuka M, Fukui Y, Ishikawa H, Lydersen C, Kovacs KM. 2002. Chemical characterization of the oligosaccharides in beluga (*Delphinapterus leucas*) and minke whale (*Balaenoptera acutorostrata*) milk. *Comparative Biochemistry and Physiology* **B132**, 611–624.
- Urashima T, Sumiyoshi W, Nakamura T, Arai I, Saito T, Komatsu T, Tsubota T. 1999b. Chemical characterization of milk oligosaccharides of the Japanese black bear, *Ursus thibetanus japonicus*. *Biochimica et Biophysica Acta* **1452**, 290–306.
- Urashima T, Yamamoto M, Nakamura T, Arai I, Saito T, Namiki M, Yamaoka K, Kawahara K. 1999a. Chemical characterization of the oligosaccharides in a sample of milk of a white-nosed coati, *Nasua narica* (Procyonidae: Carnivora). *Comparative Biochemistry and Physiology* **A123**, 187–193.
- Urashima T, Yamashita T, Nakamura T, Arai I, Saito T, Derocher AE. 2000. Chemical characterization of milk oligosaccharides of the polar bear, *Ursus maritimus*. *Biochimica et Biophysica Acta* **1475**, 395–408.