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Structural characterization of neutral and acidic oligosaccharides in the milks of strepsirrhine primates: greater galago, aye-aye, Coquerel's sifaka and mongoose lemur

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Abstract The structures of milk oligosaccharides were characterized for four strepsirrhine primates to examine the extent to which they resemble milk oligosaccharides in other primates. Neutral and acidic oligosaccharides were isolated from milk of the greater galago (Galagidae: *Otolemur crassicaudatus*), aye-aye (Daubentoniidae: *Daubentonia madagascariensis*), Coquerel's sifaka (Indriidae: *Propithecus coquereli*) and mongoose lemur (Lemuridae: *Eulemur mongoz*), and their chemical structures were characterized by ^{1}H-NMR spectroscopy. The oligosaccharide patterns observed among strepsirrhines did not appear to correlate to phylogeny, sociality or pattern of infant care. Both type I and type II neutral oligosaccharides were found in the milk of the aye-aye, but type II predominate over type I. Only type II oligosaccharides were identified in other strepsirrhine milks. α3′-GL (isoglobotriose, Gal(α1-3)Gal(β1-4)Glc) was found in the milks of Coquerel's sifaka and mongoose lemur, which is the first report of this oligosaccharide in the milk of any primate species. 2′-FL (Fuc(α1-2)Gal(β1-4)Glc) was found in the milk of an aye-aye with an ill infant. Oligosaccharides containing the Lewis x epitope were found in aye-aye and mongoose lemur milk. Among acidic oligosaccharides, 3′-*N*-acetylneuraminyllactose (3′-SL-NAc, Neu5Ac(α2-3)Gal(β1-4)Glc) was found in all studied species, whereas 6′-*N*-acetylneuraminyllactose (6′-SL-NAc, Neu5Ac(α2-6)Gal(β1-4)Glc) was found in all species except greater galago. Greater galago milk also contained 3′-*N*-glycolylneuraminyllactose (3′-SL-NGc, Neu5Gc(α2-3)Gal(β1-4)Glc). The finding of a variety of neutral and acidic oligosaccharides in the milks of strepsirrhines, as previously reported for haplorhines, suggests that such constituents are ancient rather than derived features, and are as characteristic of primate lactation is the classic disaccharide, lactose.

Keywords Milk oligosaccharides · Lactation · Primates · Strepsirrhines · Phylogeny · Sociality · Greater galago · Aye-aye · Sifaka · Lemur

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Introduction

The milks of eutherian mammals typically contain lactose as the dominant saccharide, but may contain a variety of oligosaccharides as well. It is generally believed that human milk oligosaccharides act as receptor analogues to inhibit the attachment of pathogenic microorganisms to the epithelial surface of the infant digestive tract, as prebiotics to stimulate the growth of beneficial microorganisms and as factors involved in immunomodulation [1]. However, there are large interspecific differences in both the types and amounts of milk oligosaccharides, and no universal pattern has emerged linking oligosaccharide concentration to particular aspects of maternal or infant biology. High concentrations of milk oligosaccharides have been found in some taxa with altricial offspring, such as monotremes, marsupials and some eutherian carnivores, but how important this is to the microbial environment of the neonatal digestive system, or to some aspect of postnatal development, are not known [2].

Among primates, most research has focused on human milk, but some research has been conducted on structural identification of milk oligosaccharides in other taxa [3, 4] (Fig. 1). Human milk contains a particularly diverse array of oligosaccharides, and the oligosaccharide patterns differ among individual women [5–7]. The predominant oligosaccharides during the first three days of human lactation (see Table 2 for oligosaccharide abbreviations) are 2′-FL, LNFP I, LNDFH I, and LNT [8]. These four oligosaccharides are also predominant in transitional and mature human milk [6, 9, 10]. The oligosaccharides LNFP I, LNDFH I, and LNT all include the type I unit (Gal(β1-3)GlcNAc, lacto-*N*-biose I) within their structures, which is in contrast to the oligosaccharides found in milk and colostrum of non-primate mammals, which contain predominantly, or solely, the type II unit (Gal(β1-4)GlcNAc, *N*-acetyllactosamine) [11].

Among the hominoids most closely related to humans, the chimpanzee, bonobo and orangutan secrete milk (or colostrum) containing both type I and type II oligosaccharides, but type II predominate over type I, whereas the gorilla and siamang secrete milks in which only type II oligosaccharides have been identified by structural analysis [3]. Among old world (Catatarrhini: Cercopithecidae) and new world (Platyrrhini) monkeys (see Fig. 1 for the phylogeny used in this paper), the milks of the rhesus macaque, toque macaque, hamadryas baboon and tufted capuchin contain oligosaccharides with the type II unit, but oligosaccharides containing the type I unit were not detected [4]. Reports assigning observed chromatographic or mass spectrometer peaks to type I constituents [12, 13] are inconclusive and subject to error unless backed by structural analysis [3]. Available data suggest that hominoid milks may be unique among primates in containing type I oligoaccharides, and that human milk is particularly enriched in type I. The

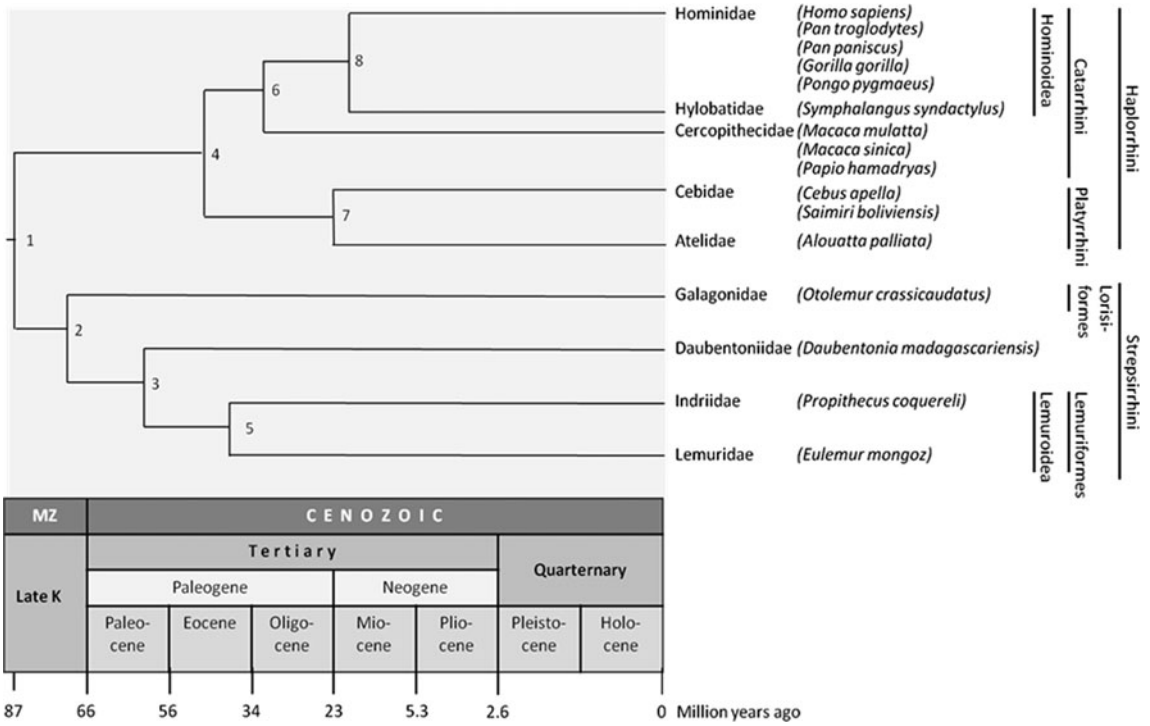

Fig. 1 Divergence of primate groups, including a listing of primate species for which oligosaccharides have been identified by ^{1}H-NMR in the current and prior publications [3, 4]. The divergence times for the nodes taken from Perelman *et al*. [15]. Figure modified from Steiper and Young [14]. *Abbreviations*: K (Cretaceous) and MZ (Mesozoic)

biological significance of a predominance of type I oligosaccharides is not clear, although we speculate that it may be important to bifidus flora formation in the infant colon (see Discussion).

Our understanding of the evolution of milk oligosaccharides in primates has been limited by a lack of information on strepsirrhine primates (*e.g.*, lorises, galagos, aye-aye, sifakas, lemurs). The separation of the strepsirrhine primates from the haplorhine lineage leading to monkeys, apes and humans occurred about 76–87 million years ago during the late Cretaceous [14, 15]. Given evidence that the array of oligosaccharides in milk becomes increasingly complex in the lineage leading to humans, we predicted that strepsirrhine milks should have few oligosaccharides, and only those containing type II units.

We obtained milk samples from four strepsirrhine species representing diverse phylogenetic lineages (Fig. 1): the greater galago representing Galagonidae within the Lorisiformes, the aye-aye, representing the monospecific Daubentoniidae (infraorder Chiromyiformes), the sifaka representing the Indriidae within the Lemuriformes, and the mongoose lemur representing the Lemuridae with the Lemuriformes. Among the Strepsirhini, the Lorisiformes diverged first, at about 69 million years ago (mya), the Daubentoniidae diverged from the Lemuriformes at about 59 mya, and the Indriidae and Lemuridae diverged about 39 million years ago [15]. Based on phylogeny we expected the oligosaccharides of the sifaka and mongoose lemur to be most similar, with the aye-aye and greater galago most disparate. However, there is also considerable variation in the social and reproductive biology of these four species, with the diurnal/cathemeral species (sifaka, lemur) living in moderately gregarious groups in which the young are carried by the mother (and sometimes others), while the noctural species (galago, aye-aye) are dispersed and relatively solitary, and park their infants in nests or tree holes while foraging (Table 1). The composition of strepsirrhine milk, including total saccharide content, has been shown to correlate with the pattern of maternal care [25]. It has also been suggested that oligosaccharide diversity in primates could be related to degree of sociality [13].

Materials and methods

Milk samples were collected from strepsirrhines maintained in breeding colonies at the Duke Lemur Center (formerly Duke University Primate Center), Durham, NC, USA. Mothers were captured, separated from their offspring and manually restrained or lightly immobilized with ketamine hydrochloride (10–15 mg) or isoflurane inhalant anesthesia (2–5%) in oxygen delivered by face mask. After IM injection of oxytocin (0.15–0.5 mL, or 3–10 IU), milk was expressed by hand. Milk samples were frozen in air tight vials at −20°C or −80°C and subsequently shipped to the Nutrition Laboratory, Smithsonian National Zoological Park, Washington DC, USA. Immediately after thawing in a warm water bath, milks were pooled within each species to give a sufficient amount (5–10 ml) for oligosaccharide analysis. The pooled samples were as follows:

1. Greater Galago (Galagidae: *Otolemur crassicaudatus*): 17.3 mL milk was obtained by pooling milk from four females. Three females were milked on 21 May 1990, 24 May 1990, and 9 February 1990 at 19, 19 and 96 days postpartum, and the fourth female was milked on 18 December 1989, 3 January 1990, and 19 January 1990, at 44, 59 and 75 days postpartum. Given a weaning age of 4.4 months (Table 1), this pooled sample represents early to mid-lactation.
2. Aye-aye (Daubentoniidae: *Daubentonia madagascariensis*): 7.1 mL mature milk was obtained by pooling milk from four females. These females were milked on 24 September 2001, 21 December 2001 and 26 June 2002 (n=2) at 56, 57, 244 and 294 days postpartum, or mid to late lactation. A pooled sample of milk (5.8 mL) was also obtained from a female on 19 December 2000 (n=1) and 20 December 2000 (n=3), at 4 and 5 days postpartum, but as the infant was ill and died on 20 December 2000, this sample may represent mammary involution and cannot be considered representative of normal lactation.
3. Sifaka (Indriidae: *Propithecus coquereli*): 7.0 mL milk was obtained by pooling samples from 4 females. Milk

Table 1 Reproductive and behavioral characteristics of the species studied (references [16–24])

Species	Common name	Female body mass (kg)	Age at weaning (mo)	Activity Pattern	Social spacing	Group size (not including infant)	Care type
Otolemur crassicaudatus	Greater galago	1.2	4.4	Nocturnal	Dispersed[a]	1[a]	Park in nest/tree hole
Daubentonia madagascariensis	Aye-aye	2.6	8.8	Nocturnal	Dispersed	1	Park in nest
Propithecus coquereli	Coquerel's sifaka	4.1	6.0	Diurnal	Gregarious	5 (2–8)	Carry
Eulemur mongoz	Mongoose lemur	1.6	5.0	Cathemeral	Gregarious	3 (2–4)	Carry

[a] sometimes adult pairs sleep with offspring

was collected from one female on 28 April 1999, 21 May 1999 and 9 May 2000, at 63, 86 and 97 days postpartum and from three other females at 21 April 1999, 26 June 2002 and 16 March 1999 at 65, 73 and 87 days postpartum. This pooled sample represents mid-lactation.

4. Mongoose Lemur (Lemuridae: *Eulemur mongoz*): 9.4 g milk was collected from one female on 27 April 1988, 6 May 1988 and 3 June 1988 at 25, 36 and 62 days postpartum, from a second female on 6 May 1988, 17 May 1988, 3 June 1988 and 26 June 1988, at 30, 41, 58 and 81 days postpartum, and from a third female on 20 June 1988, at 56 days postpartum. This pooled sample represents mid-lactation.

After extraction and lyophilization to recover total carbohydrate, the dried extracts were shipped to Obihiro University of Agriculture and Veterinary Medicine, Japan for further analysis.

Oligosaccharide reference materials (see Table 2 for abbreviations), LNFP II, LNFP III, LNFP V, LNT, LNnT, LNnH, LSTc, 2′-FL, and 3-FL, were purchased from Seikagaku Co. (Tokyo, Japan), whereas bovine disialyllactose (Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc), 3′-NAc-SL and 6′-NAc-SL were obtained from Sigma Co. (St. Louis, MO, USA). B-Tetrasaccharide was isolated from Japanese black bear milk [26], while 3′-NGc-SL was isolated from ovine colostrum [27]. β6′-GL and β3′-GL were purified from caprine colostrum [28]. MSLNnH, MSMFLNnH, DFLNnH and lactose 3′-O-sulfate were isolated from siamang milk, orangutan colostrum, orangutan colostrum [3] and dog milk [29], respectively. Isoglobotriose (Gal(α1-3)Gal(β1-4)Glc) was purified from caprine colostrum [28], giant anteater [30] and Asian elephant [31].

Milk samples were thawed, pooled, and extracted with four volumes of chloroform/methanol (2:1, v/v). After agitation, the emulsion was centrifuged at 5000× g at 4°C for 30 min, and the lower chloroform layer and the denatured protein residue were discarded. The methanol was evaporated from the upper layer, and the lyophilized residue was designated as the carbohydrate fraction.

The carbohydrate fraction from each sample was dissolved in 2 mL water, and passed through a BioGel P-2 (<45 μm, 2.5×100 cm, Bio-Rad, Hercules, CA) that had been calibrated with 2 mg each of galactose (monosaccharide), lactose (disaccharide) and raffinose (trisaccharide).

Table 2 Milk oligosaccharides characterized in this study (* = type I, ^ = type II)

Oligosaccharide (abbreviation)	Chemical structure
2′-Fucosyllactose (2′-FL)	Fuc(α1-2)Gal(β1-4)Glc
3-Fucosyllactose (3-FL)	Gal(β1-4)[Fuc(α1-3)]Glc
3′-Galactosyllactose (β3′-GL)	Gal(β1-3)Gal(β1-4)Glc
Isoglobotriose (α3′-GL)	Gal(α1-3)Gal(β1-4)Glc
6′-Galactosyllactose (β6′-GL)	Gal(β1-6)Gal(β1-4)Glc
Difucosyllactose (DFL)	Fuc(α1-2) Gal(β1-4)[Fuc(α1-3)]Glc
Lacto-*N*-tetraose (LNT)*	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
Lacto-*N*-neotetraose (LNnT)^	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
B-Tetrasaccharide (B-tetra)	Gal(α1-3)[Fuc(α1-2)]Gal(β1-4)Glc
Lacto-*N*-fucopentaose II (LNFP II)*	Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Gal(β1-4)Glc
Lacto-*N*-fucopentaose III (LNFP III)^	Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc
Lacto-*N*-fucopentaose VI (LNFP VI) ^	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]Glc
Lacto-*N*-neohexaose (LNnH)^	Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]-Gal(β1-4)Glc
Fucosyl-lacto-*N*-neohexaose a (FLNnH a)^	Gal(β1-4)GlcNAc(β1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc
Fucosyl-lacto-*N*-neohexaose b (FLNnH b)^	Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3){Gal(β1-4)GlcNAc(β1-6)]}Gal(β1-4)Glc
Difuco-lacto-*N*-neohexaose (DFLNnH)^	Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc
3′-Neu5AcLac (3′- SL-NAc)	Neu5Ac(α2-3)Gal(β1-4)Glc
3′-Neu5GcLac (3′-SL-NGc)	Neu5Gc(α2-3)Gal(β1-4)Glc
6′-Neu5AcLac (6′-SL-NAc)	Neu5Ac(α2-6)Gal(β1-4)Glc
Disialyllactose (DSL)	Neu5Ac(α2-3)Gal(β1-4) [Neu5Ac(α2-6)]Glc
Sialyllacto-*N*-tetraose c (LSTc)	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
Monosialyl-monofucosyl-LNnH (MSMFLNnH)	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc
Monosialyl-LNnH (MSLNnH)	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc
3-O-lactose sulfate (L-3′-s)	Gal(β1-4)Glc-3′-O-sulfate

The gel was washed with 0.1 M NaOH and 0.1 M HCl before use. Elution was done with distilled water at a flow rate of 15 mL/h, and 5 mL fractions were analyzed for hexose with the phenol-H_2SO_4 method [32] and for sialic acid with the periodate-resorcinol method [33] (chromatograms in Figs. 2 and 3). The peak fractions were pooled and freeze dried. The components in each peak were analyzed by thin layer chromatography (TLC) on Silica Gel 60 (20× 20 cm; Merck, Darmstadt, Germany) with acetone/2-propanol/0.1 M lactic acid (2:2:1, v/v/v) as a developing solvent. Spots were detected by spraying with 5% sulfuric acid in 99.5% ethanol and heating above a flame. The components

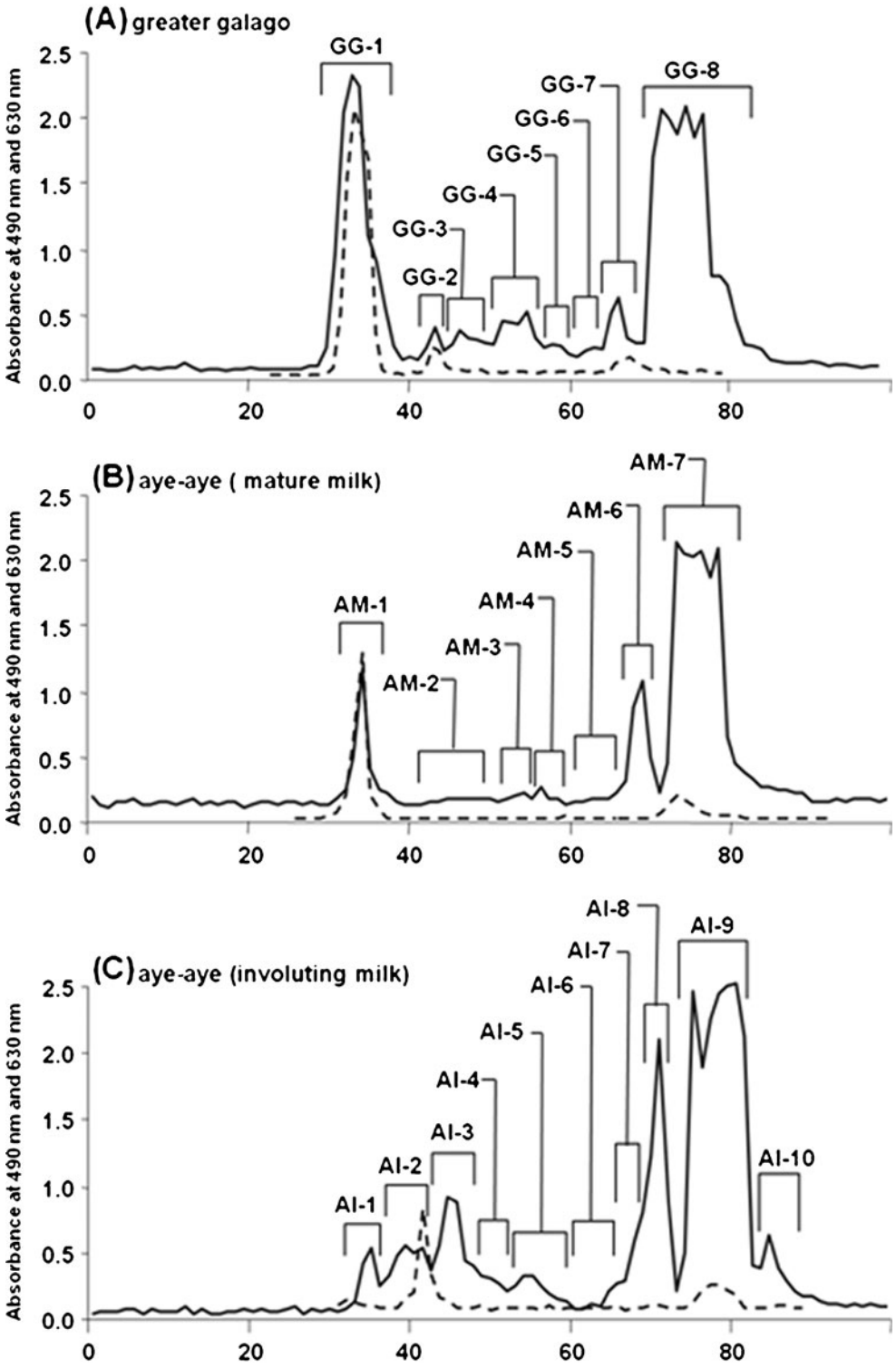

Fig. 2 Gel chromatograms of the carbohydrate fraction from milk of (**a**) greater galago, (**b**) aye-aye (mature milk) and (**c**) aye-aye (involuting milk). Elution from a BioGel P-2 column (2.5×100 cm) was done with distilled water at a flow rate of 15 mL/h, and of 5.0 mL fractions were collected. Each fraction was monitored by the phenol-H_2SO_4 method at 490 nm (as shown by the solid line) and the periodate-resorcinol method at 630 nm (as shown by the dotted line)

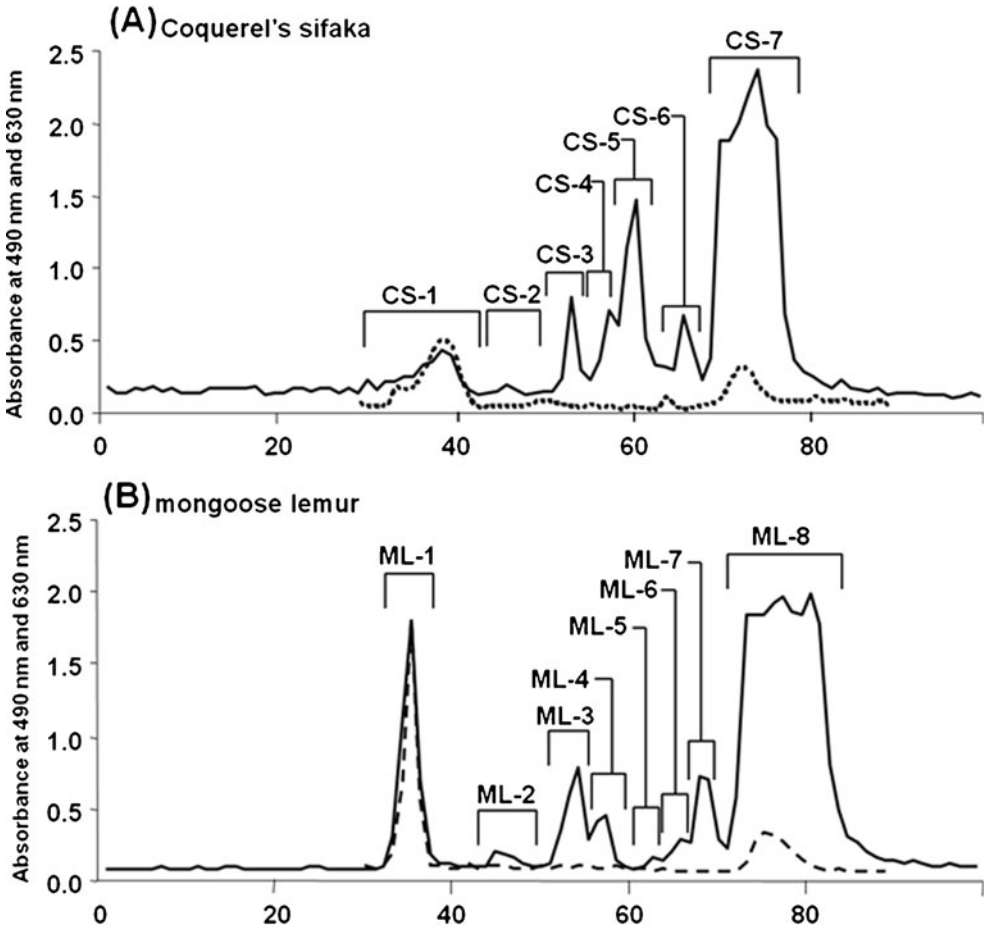

Fig. 3 Gel chromatograms of the carbohydrate fraction from milk of (**a**) Coquerel's sifaka and (**b**) mongoose lemur. Gel chromatography was done as in Fig. 2

in GG-2, GG-3, GG-4, GG-5, GG-6, GG-7 and GG-8 from greater galago milk (Fig. 2a), AM-2, AM-3, AM-4, AM-5. AM-6 and AM-7 from aye-aye mature milk (Fig. 2b), AI-3, AI-4, AI-5, AI-6, AI-7, AI-8, AI-9 and AI-10 from aye-aye involuting milk (Fig. 2c), CS-2, CS-3, CS-4, CS-5, CS-6 and CS-7 from Coquerel's sifaka milk (Fig. 3a), ML-2, ML-3, ML-4, ML-5, ML-6, ML-7 and ML-8 from mongoose lemur milk (Fig. 3b) were characterized by ^{1}H-NMR spectroscopy to characterize their chemical structures.

Fractions GG-1 (Fig. 2a), AM-1 (Fig. 2b), AI-2 (Fig. 2c) and ML-1 (Fig. 3b) from the greater galago milk, aye-aye mature milk, aye-aye involuting milk, and mongoose lemur milk, respectively, were dissolved in 2 ml of 50 mM Trishydroxyaminomethane-HCl buffer (pH 8.7) and subjected to anion-exchange chromatography using a DEAE-Sephadex A-50 (GE Healthcare, Uppsala, Sweden) column (2.0×20 cm) equilibrated with the same buffer solution. The unadsorbed components were eluted with 250 mL of the same buffer solution. Elution was done 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 using the phenol-H_2SO_4 method. The peak fractions, designated as GG-1-1, AM-1-1, AI-2-1 and ML-1-1, were each pooled, lyophilized, dissolved in 2 mL of water and passed through a column (2.0×35 cm) of BioGel P-2 to remove salts, as described above.

Each component in GG-1-1 (Fig. 4a), AM-1-1 (Fig. 4b), AI-2-1 (Fig. 4c), CS-1 (Fig. 5a) and ML-1-1 (Fig. 5b) was further purified using 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, Kyoto, Japan) (see chromatograms in Figs. 4 and 5). The mobile phase was 50% and 80% (v/v) acetonitrile (CH_3CN) in a 15 mM potassium phosphate buffer (pH 5.2). Elution was done using a linear gradient of acetonitrile, 80–50% 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 characterized by ^{1}H-NMR.

The NMR spectra were recorded in D_2O (100.00 atom% D, Aldrich, Milwaukee, USA) at 500 or 600 MHz for ^{1}H-NMR with a JEOL-ECP-500 FT-NMR or Varian INOVA 600 spectrometer operated at 293.1 K. Chemical shifts are expressed in ppm downfield from internal 3-(trimethylsilyl)-1-propane sulfonic acid, sodium salt (TPS), but were actually measured by reference to internal acetone (δ=2.225).

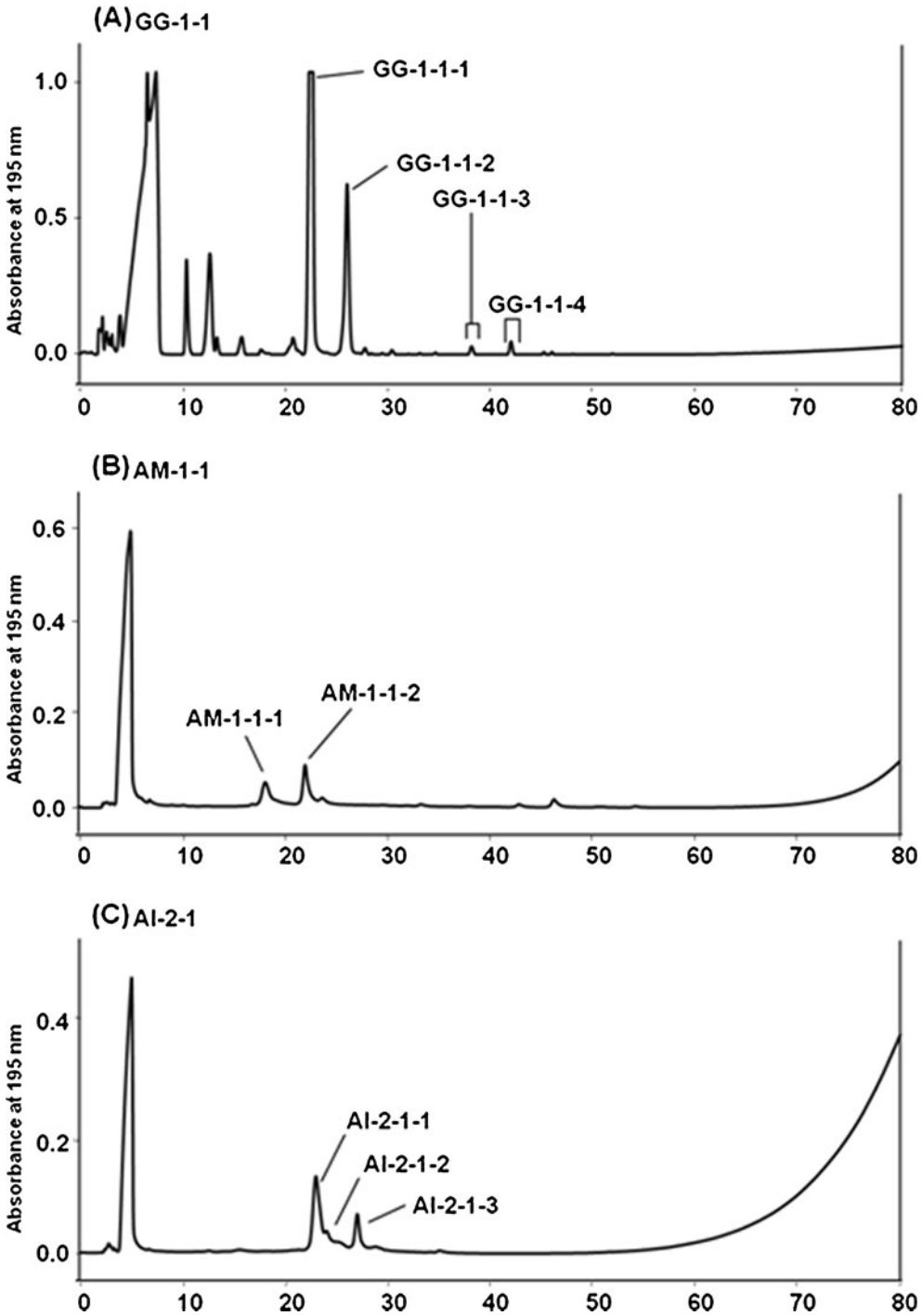

Fig. 4 HPLC of (**a**) GG-1-1, (**b**) AM-1-1 and (**c**) AI-2-1 separated from greater galago, aye-aye mature milk and aye-aye involuting milk, respectively. HPLC was done using a Shimadzu LC-10 AT VP pump on a TSK-gel Amido-80 column (4.6× 250 mm, pore size 80 Å, particle size 5 μm). The mobile phase was 80% and 50% acetonitrile in 15 mM potassium phosphate buffer solution, denoted buffer A and buffer B. Elution was done using a linear gradient of acetonitrile from 80% to 50% at 60°C at a flow rate of 1 mL/min. Peaks were detected from UV absorption at 195 nm

Results

Following size-exclusion chromatography on BioGel P-2, the carbohydrate fraction of each sample of milk or colostrum was resolved into multiple peaks, as shown in Figs. 2 and 3. Six to eight chromatographic peaks from each pooled sample were characterized by ^{1}H-NMR. Peaks, which reacted positively for sialic acid were found after anion-exchange chromatography to contain just one peak each (chromatograms not shown), designated as GG-1-1, AM-1-1, AI-2-1 and ML-1-1 for greater galago milk, aye-aye mature milk, aye-aye involuting milk and mongoose lemur milk, respectively. These crude sialyl oligosaccharide fractions were passed through a BioGel P-2 column and each oligosaccharide was separated by HPLC (chromatograms in Figs. 4 and 5) prior to characterization by ^{1}H-NMR. Fraction CS-1 (Fig. 3a) from Coquerel's sifaka milk was not subjected to anion-exchange chromatography since the

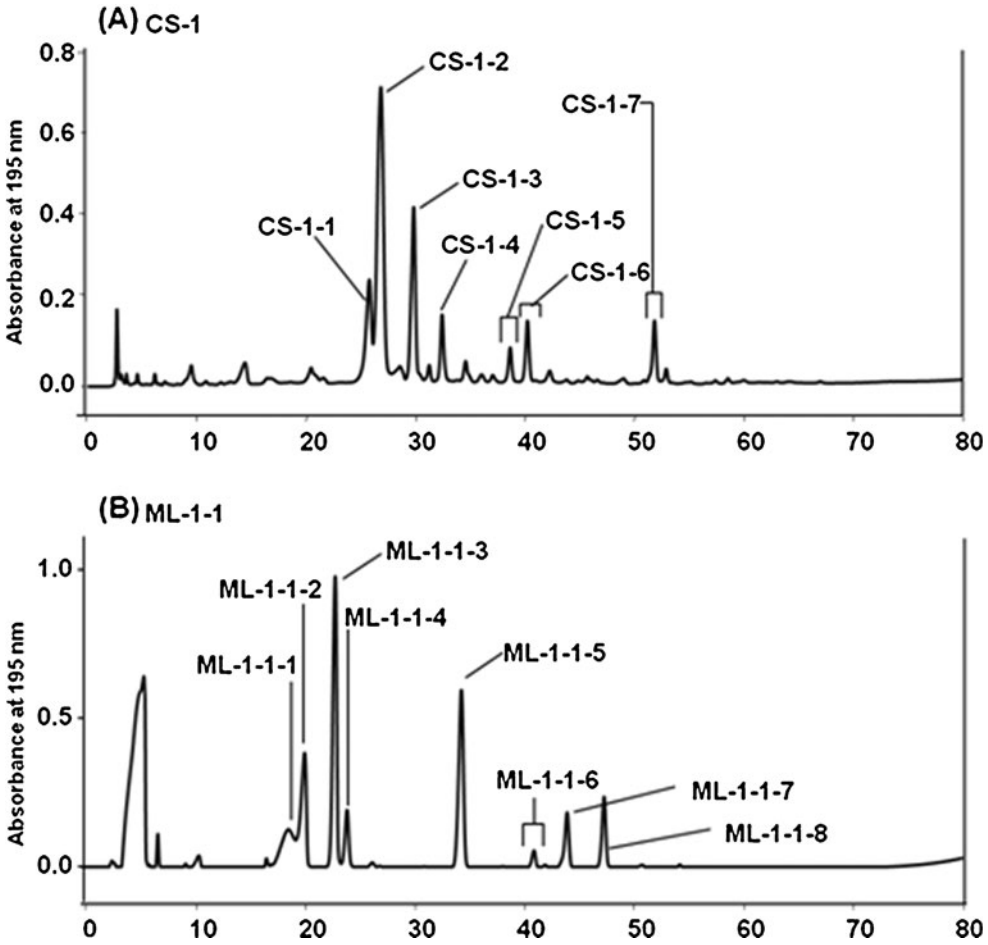

Fig. 5 HPLC of (**a**) CS-1 and (**b**) ML-1-1 separated from Coquerel's sifaka and mongoose lemur milk, respectively. HPLC was performed as described in Fig. 4

amount of pooled material was too small; it was rather purified directly by HPLC analysis (Fig. 5a) and then characterized by ^{1}H-NMR.

The structures and abbreviations for oligosaccharides characterized in this study are listed in Table 2. Examples of ^{1}H-NMR spectra (Figs. S1–S4) and tabulated data on the chemical shifts for ^{1}H-NMR spectra (S1–S13) are presented as supplementary information.

Greater galago milk

Acidic oligosaccharides

The ^{1}H-NMR spectra (Table S1) of GG-1-1-1 and GG-1-1-2 (Fig. S1) were essentially identical with those of 3′-NAc-SL and 3′-NGc-SL (see reference [27]), respectively; other minor peaks in Fig. 4a could not be characterized because the amounts were insufficient.

Neutral oligosaccharides

The three components (GG-4, GG-7 and GG-8) had ^{1}H-NMR spectra (Table S2) that were identical with those of LNnT, β6′-GL and lactose. The components in four small peaks (GG-2, GG-3, GG-5 and GG-6; Fig. 2a) could not be characterized in this study.

Aye-aye milk

Acidic oligosaccharides

The ^{1}H-NMR chemical shifts (Table S3) of AM-1-1-1 and AM-1-1-2 were identical to those of 3′-NAc-SL and 6′-NAc-SL.

Neutral oligosaccharides

The neutral oligosaccharide peaks obtained from aye-aye milk contained both single components (AM-5, AM-6 and AM-7) and mixtures of components (AM-2, AM-3 and AM-4).

The ^{1}H-NMR chemical shifts (Table S4) of AM-2 were similar to those of ML-2 (see below), indicating an unresolved mixture of LNnH and its fucosyl derivatives. The spectrum had: 1. The anomeric shifts of α-Glc, β-Glc, β(1-3) linked GlcNAc, two β(1-6) linked GlcNAc and three

β(1-4) linked Gal at δ 5.218, 4.666, 4.699, 4.637 and 4.644, 4.427, 4.471 and 4.481, respectively; 2. The H-4 shift of β(1-4) linked Gal, in which OH-3 position was substituted by GlcNAc residue via β-linkage, at δ 4.148; 3. NAc shifts of β(1-3) and β(1-6) linked GlcNAc at δ 2.030 and 2.061, respectively. These were identical to chemical shifts of the LNnH standard. However, the spectrum also had two H-1 shifts of α(1-3) linked Fuc at δ 5.105 and 5.125, two H-1 shifts of β(1-4) linked Gal at δ 4.455 and 4.466, and H-6 shifts of α(1-3) linked Fuc at δ 1.174. The spectrum also had other NAc shifts of β(1-3) and β(1-6) linked GlcNAc at δ 2.019 and 2.053, respectively, which would have moved upfield from δ 2.030 and 2.061 by the substitution of α(1-3) linked Fuc in these residues. These shifts are essentially similar to published data of DFLNnH [34]. However, these data are not definitive for LNnH and DFLNnH, but could also include monofucosyl form (FLNnH) in one or two isomeric forms. The ratio of non-fucosylation to fucosylation of β(1-3) linked GlcNAc and of β(1-6) linked GlcNAc were estimated to be 1 : 0.8 and 1 : 1.1 from the relative signal intensities of δ 2.030 vs 2.019, and of δ 2.061 vs 2.053, respectively.

The ^{1}H-NMR spectra of AM-3 (chemical shifts in Table S5) indicated the presence of three oligosaccharides, AM-3-1, AM-3-2 and AM-3-3:

1. AM-3-1 had the anomeric shifts of α-Glc, β-Glc, β(1-4) linked Gal, β(1-3) linked Gal, α(1-4) linked Fuc, β(1-3) linked GlcNAc, H-4 of β(1-4) linked Gal [which was substituted at OH-3 by a β-linked GlcNAc], H-5 of α(1-4) linked Fuc, H-6 of α(1-4) linked Fuc and NAc of β(1-3) linked GlcNAc at δ 5.219, 4.663, 4.434, 4.506, 5.029, 4.695, 4.158, 4.881, 1.179 and 2.032, respectively. This pattern was virtually identical to the reference standard of LNFP II.
2. The ^{1}H-NMR spectrum of AM-3-2 had the six anomeric shifts of the H-1 signal of α-Glc, α(1-3) linked Fuc, β(1-3) linked GlcNAc, β-Glc and two β(1-4) linked Gal at δ 5.219, 5.128, 4.707, 4.663, 4.463 and 4.434, respectively; it also had the H-4 signal of β(1-4) linked Gal, which was substituted at OH-3 by a β-linked GlcNAc at δ 4.158, the NAc shift of β(1-3) linked GlcNAc at δ 2.022 and the H-6 of α(1-3) linked Fuc at δ 1.174. Thus AM-3-2 is essentially identical with LNFP III, as well as published data for this compound in fractions R2 in rhesus macaque milk and T4 of toque macaque milk [4].
3. The ^{1}-NMR spectrum for AM-3-3 had the anomeric shifts of α(1-3) linked Fuc linked to non reducing Glc at δ 5.429 and 5.372, H-1 shifts of β(1-3) linked GlcNAc and two β-linked Gal at δ 4.707, 4.434 and 4.413, respectively; and NAc shift of β(1-3) linked GlcNAc at δ 2.028. We considered two possible structures, Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]Glc (lacto-N-fucopentaose V; LNFP V, type I) and Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]Glc (type II). However, the ^{1}H-NMR spectra of LNFP V standard had the H-1 shifts of β(1-3) linked GlcNAc, β(1-3) linked Gal and β(1-4) linked Gal at δ 4.718, 4.448, 4.418, respectively, and the NAc shift of β(1-3) linked GlcNAc at δ 2.023, indicating that AM-3-3 is not the same as LNFP V. Moreover, the H-4 shift (δ 4.158) of β(1-4) linked Gal, which was substituted at OH-3 by a β-linked GlcNAc, and two H-6 shifts (δ 1.177 and 1.174) of α(1-3) linked Fuc, were shifted down field, as compared to those of LNFP V (δ 4.098, 1.165, 1.159, respectively). From these observations, the oligosaccharide in AM-3-3 was characterized as Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]Glc, which was designated as lacto-*N*-fucopentaose VI (LNFP VI).
4. The estimated ratio of LNFP-II (AM-3-1) to LNFP III (AM-3-2) based on their signal intensities of H-1 of α(1-4) and α(1-3) linked Fuc was about 1:1.5.

The ^{1}H-NMR spectra of AM-4 (Table S6) showed that it contained two oligosaccharides, one minor (AM-4-1) and the other major (AM-4–2). The spectra of the two oligosaccharides were essentially identical with those of the standards LNT and LNnT. The ratio of LNT to LNnT was estimated to be about 1:1.5 from the signal intensities of NAc for both β(1-3)-linked GlcNAc residues.

The ^{1}H-NMR spectrum of AM-5 (Table S6) had the anomeric shifts of α-Glc, β-Glc, β(1-4) linked Gal, α(1-2) linked Fuc, two of α(1-3) linked Fuc, H-6 of α(1-2) linked Fuc and α(1-3) linked Fuc at δ 5.175, 4.622, 4.490, 5.283, 5.451 and 5.467, 1.266 and 1.255, respectively. From this pattern, the oligosaccharide was identifed as DFL.

The spectra of AM-6 and AM-7 were essentially identical with those of 3-FL and lactose, respectively.

Involuting sample

A pooled sample of milk from an apparently involuting gland was examined. The ^{1}H-NMR chemical shifts of AI-2-1-1 and AI-2-1-2 (Table S7) were both essentially identical to 3′-NAc-SL, whereas AI-2-1-3 was essentially identical to 6′-NAc-SL. The ^{1}H-NMR spectra of AI-7, AI-8 and AI-9 (Table S8) were identical with those of 2′-FL, 3-FL and lactose, respectively. The gel chromatogram in Fig. 2c showed that peak AI-1 eluted before AI-2, which has been identified as acidic oligosaccharides. Based on this observation, we conclude that peak AI-1 is not an oligosaccharide but rather a glycopeptide/glycoprotein compound, which we did not attempt to characterize. The identities of various small peaks, AI-3, AI-4, AI-5 and AI-6 (Fig. 2c), could not be determined in this study, because their ^{1}H-NMR spectra were unusual. AI-10 proved to be the monosaccharide glucose based on its mobility on thin layer chromatography (TLC).

Coquerel's sifaka milk

Acidic oligosaccharides

As the ^{1}H-NMR spectra of CS-1-1 and CS-1-2 (Table S9) were similar, both were identified as 3′-NAc-SL, whereas CS-1-3 was identical with 6′-NAc-SL. The identities of CS-1-4 and CS-1-5 peaks could not be determined in this study due to their unusual ^{1}H-NMR spectra.

The spectrum of CS-1-6 (Table S9) had two H-3 axial signals of Neu5Ac at δ 1.797 and 1.725, suggesting that the component in this peak had two Neu5Ac residues. The signal intensity of H-3 equatorial of Neu5Ac at δ 2.703 was twice that of the two H-3 axial signals. The spectrum had the H-1 signals of α-Glc, β-Glc and β(1-4) linked Gal at δ 5.223, 4.672 and 4.492, respectively. From these observations, we suggest that the component is a disialyllactose, but the NMR pattern differed from that of (Neu5Ac(α2-8)Neu5Ac(α2-3) Gal(β1-4)Glc), which has been separated from bovine colostrum. We hypothesize that the structure of this disialyllactose is Neu5Ac(α2-3) Gal(β1-4)[Neu5Ac(α2-6)] Glc.

The spectrum of CS-1-7 (Fig. S2, Table S9) had the anomeric shifts of α-Glc, β(1-3) linked GlcNAc, β-Glc, two β(1-6) linked GlcNAc, and three β(1-4) linked Gal at δ 5.221, 4.734, 4.669, 4.651 and 4.640, and 4.472, 4.452 and 4.437, respectively. The spectrum had the characteristic H-3 axial and H-3 equatorial of α(2-6) linked Neu5Ac at δ 1.724 and 2.671, respectively; the NAc of α(2-6) linked Neu5Ac, β(1-6) and β(1-3) linked GlcNAc at δ 2.027, 2.051 and 2.061, respectively; and H-4 of β(1-4) linked Gal, which was substituted at OH-3 by β-GlcNAc, at δ 4.147. This pattern was equivalent to published data for monosialyl-LNnH (MSLNnH) [35], and the pattern found when this oligosaccharide was isolated (as S-1-4) from siamang milk [3].

Neutral oligosaccharides

The ^{1}H-NMR spectrum (Table S10) of CS-2 was essentially identical to that of the standard LNnH. The spectrum of CS-4 includes anomeric shifts at δ 5.225, 4.666 and 4.568, a characteristic doublet doublet shift at δ 4.343, and a doublet shift at δ 4.294, which are virtually identical to anomeric shifts of α-Glc, β(1-4) linked Gal, and H-3 and H-4 of β(1-4) linked Gal in 3′-*O*-lactose sulfate, respectively [29]. Thus CS-4 was identified as 3′-*O*-lactose sulfate. The ^{1}H-NMR spectra of CS-5 (Table S10) indicated that it contained two oligosaccharides, CS-5-1 and CS-5-2. Based on similarity of its ^{1}H-NMR spectra to published data [26] CS-5-1 was identified as B-tetrasaccharide. As the signals of fraction CS-5-2 were identical to those of CS-4 and to data previously published on 3′-*O*-lactose sulfate [29], it was identified as Gal(β1-4)Glc-3′-O-sulfate.

The spectrum of CS-6 (Table S10) had the four anomeric shifts of α-Glc, β-Glc, α(1-3) and β(1-4) linked Gal at δ 5.225, 4.669, 5.145 and 4.525, respectively. The spectrum also had signals of H-4 of β(1-4) linked Gal and of H-5 of α(1-3) linked Gal at δ 4.184 and 4.197, respectively. These chemical shifts were similar to published data for the Mt5 fraction of giant anteater milk [30] and the Em2 fraction of Asian elephant milk [31], which were identified as isoglobotriose; thus CS-6 was also identified as isoglobotriose. The ^{1}H-NMR spectra (Table S10) of CS-7 was essentially identical to lactose. The identity of the CS-3 peak (Fig. 3a) was not determined.

Mongoose lemur milk

Acidic oligosaccharides

The ^{1}H-NMR spectra of ML-1-1-1 and ML1-1-2 (Table S11) were both identical to 3′-NAc-SL, whereas ML-1-1-3 and ML-1-1-5 were identical to 6′-NAc-SL, and LSTc, respectively. The chemical shift data of these fractions were also identical with oligosaccharide fractions 3′-NAc-SL (peak S-1-1), 6′-NAc-SL (S-1-2) and LSTc (S-1-3) of siamang milk [3]. The ^{1}H-NMR spectrum of ML-1-1-7 demonstrated the same pattern of anomeric shifts as CS-1-7 of sifaka milk and was also very similar to published data [3] of siamang S-1-4 fraction, previously identified as MSLNnH. Thus this oligosachharide was identified as the heptasaccharide MSLNnH. The ^{1}H-NMR spectrum of ML-1-1-8 had the anomeric shifts of α-Glc, β(1-3)-linked GlcNAc, β-Glc, β(1-6)-linked GlcNAc, H-1 and H-6 α(1-3) linked Fuc, and three β(1-4)-linked Gal at δ 5.218, 4.725, 4.668, 4.639, 5.104 and 1.171, and 4.453, 4.453 and 4.432, respectively. The spectrum also had the characteristic H-3 axial, H-3 equatorial, and NAc of α(2-6)-linked Neu5Ac at δ 1.723, 2.668, and 2.027, respectively; the NAc of β(1-3) and β(1-6)-linked GlcNAc at δ 2.051 and 2.061, respectively; and H-4 of β(1-4)-linked Gal, which was substituted at OH-3 by β-GlcNAc, at δ 4.143. As this pattern was essentially similar to MSMFLNnH, the oligosaccharide in ML-1-1-8 was identified as this octasaccharide. Based on their ^{1}H-NMR spectra, the other minor peak components shown in Fig. 5b, were not saccharides.

Neutral oligosaccharides

The neutral oligosaccharide peaks obtained from mongoose lemur milk contained both single components (ML-3, ML-4 and ML-8) and mixtures of components (ML-2 and ML-7).

The ^{1}H-NMR spectrum of ML-2 (Table S12; Figs. S3 and S4) was similar to that of AM-2 (see above), indicating that this fraction also contained LNnH and its fucosyl derivatives. The spectrum had the anomeric shifts of α-Glc, β-

Glc, β(1-3) linked GlcNAc, β(1-6) linked GlcNAc and three β(1-4) linked Gal at δ 5.218, 4.664, 4.697, 4.636 and 4.640, 4.425, 4.472 and 4.478, respectively. The spectrum also had the H-4 shift of β(1-4) linked Gal, in which OH-3 position was substituted by GlcNAc residue via β-linkage, at δ 4.148, and NAc shifts of β(1-3) and β(1-6) linked GlcNAc at δ 2.031 and 2.060, respectively. These were identical with those of the LNnH standard. However, the spectrum also had two H-1 shifts of α(1-3) linked Fuc at δ 5.105 and 5.125, and two H-1 shifts of β(1-4) linked Gal at δ 4.452 and 4.466, and H-6 shifts of α(1-3) linked Fuc at δ 1.174. The spectrum also had NAc shifts of β(1-3) and β(1-6) linked GlcNAc at δ 2.020 and 2.051, respectively; these would be expected to have moved upfield from δ 2.031 and 2.060 by the substitution of α(1-3) linked Fuc of these residues. This pattern of shifts is equivalent to published data on DFLNnH [34] and to that of fraction O-2 from orangutan colostrum [3]. As in the case of AM-2, these data indicate the presence of LNnH and DFLNnH in this fraction, but could also include FLNnH in two isomeric forms. We conclude that ML-2 represents a possible mixture of LNnH, FLNnH and DFLNnH. The ratio of non-fucosylation to fucosylation of β(1-3) linked GlcNAc and β(1-6) linked Glc was estimated to be 1 : 1.01 and 1 : 1.34, respectively from the relative signal intensities of δ 2.031 vs. 2.020, and of δ 2.060 vs 2.051.

The ^{1}H-NMR chemical shifts (Table S13) of ML-3, ML-4 and ML-8 were essentially identical with those of AM-3-2 and AM-4-2 of aye-aye milk and that of the lactose standard, and were therefore considered to be LNFP III, LNnT and lactose, respectively.

The ^{1}H-NMR spectra of ML-7 (Table S13) showed that it contained three oligosaccharides, ML-7-1, ML-7-2 and ML-7-3. The amomeric shifts of these three oligosaccarides were essentially identical with those of 3-FL, β3′-GL and α3′-GL (isoglobotriose), respectively. Note that ML-7-1 and ML-7-3 had the same anomeric shift patterns as AI-8 of aye-aye involuting milk and CS-6 of sifaka milk. Two small peaks, ML-5 and ML-6 (Fig. 3b), could not be characterized in this study, because the chemical shift intensities were small.

Discussion

Amount of oligosaccharide

All of the assayed strepsirrhine milks included substantial amounts of oligosaccharides. The four species represent all three strepsirrhine infraorders (Lorisiformes [galago], Chiromyiformes [aye-aye] and Lemuriformes [sifaka, lemur]) and four of the seven strepsirrhine families; only Lorisidae [lorises], Cheirogaleidae [dwarf and mouse lemurs] and Lepilemuridae [sportive lemurs] were not sampled. Thus it appears that secretion of milk oligosaccharides is typical of strepsirrhines.

The relative amounts of milk oligosaccharides and lactose in the strepsirrhine milks could be estimated from the peak areas of the gel chromatograms shown in Figs. 2 and 3. The estimated ratios of oligosaccharide:lactose were: greater galago 1:1.5, aye-aye 1:3, sifaka 1:3 and mongoose lemur 1:3. These ratios were similar to reported values for most hominoid milks (chimpanzee 1:4, bonobo 1:5, orangutan colostrum 1:0.8, siamang 1:3, but gorilla colostrum 1:20) [3] and old world monkeys in the Cercopithecidae (rhesus macaque 1:6, toque macaque 1:4, hamadrys baboon 1:4) [4], but were considerably higher than in new world monkeys or Platyrrhini (tufted capuchin 1:13, Bolivian squirrel monkey 1:23, mantled howler 1:12) [4].

The factors that drive the evolution of oligosaccharide concentrations, either in total or in relation to milk lactose, are not certain. Based on phenol-sulfuric acid analysis of whole milk, the total saccharide content of mid-lactation milk are: greater galago 6.4%, aye-aye 5.8%, Coquerel's sifaka 4.1% and mongoose lemur 7.9% (Tilden and Oftedal [25], Oftedal and Williams, unpublished data). Thus strepsirrhine milks are tentatively estimated to contain about 2.5%, 1.5%, 1.0% and 2.0% oligosaccharide in greater galago, aye-aye, sifaka and mongoose lemur, respectively.

Synthesis of oligosaccharides permits secretion of isoosmotic milks that have a greater mass of saccharide per unit volume than does lactose synthesis. In the absence of other solutes, the isoosmotic lactose concentration is 110 g/L water [36] but there is an inverse relationship between lactose and other osmolytes (such as sodium, potassium and chloride). In species without significant amounts of oligosaccharides, the maximal total saccharide concentration appears to be about 7% by mass, as seen in perissodactyls [37]. Oligoaccharides permit lemurids to produce milk with somewhat higher total saccharide concentrations of 7.7–8.9% [25]. Lorisid and galagid milks presumably also contain oligosaccharides, as we observed in the greater galago, but contain less total saccharide (4.2–7.1%) [25].

Given that milks of both strespirrhine and haplorhine primates (Urashima *et al.* [3], Goto *et al* [4]) contain oligosaccharides, we conclude that secretion of milk oligosaccharides is a plesiomorphic character inherited from a Cretaceous ancestor predating the strepsirrhine-haplorhine split (Fig. 1). Based on oligosaccharide:lactose ratios of extant species, we hypothesize that the common ancestor of extant primates produced milk with a substantial proportion of oligosaccharides, and that this proportion subsequently declined in platyrrhines. Alternatively, there could have been a parallel increase in milk oligosaccharides during the evolution of strepsirrhines and catarrhines (including apes).

The diversity of milk oligosaccharides

The four strepsirrhine species varied considerably in the numbers of individual milk oligosaccharides. We identified

4 oligosaccharides in greater galago milk, 13 in aye-aye milk, 8 in Coquerel's sifaka milk, and 13 in mongoose lemur milk (Table 3). All of the acidic oligosaccharides and most of the neutral oligosaccharides that we identified were isolated as single peaks, but in a few cases a mixture of fucosylated oligosaccharides had to be teased apart, leaving some uncertainty (see Results; Table 3). For purposes of comparison, we assumed that aye-aye and mongoose lemur contained LNnH and its mono-fucosyl (FLNnH) and di-fucosyl (DFLNnH) forms. On phylogenetic grounds, we expected the greatest similarity between the milks of the sifaka and lemur, representing the two taxa (Lemuridae, Indriidae) that had diverged most recently (Fig. 1, ca. 39 mya [15]), but the sifaka and lemur shared only five identified milk oligosaccharides. By contrast, the aye-aye and lemur, representing taxa (Chiromyiformes and Lemuriformes) that diverged about 59 mya, shared 8 identified milk oligosaccharides (Table 3).

Only a small number of oligosaccharides were identified in greater galago milk, despite the larger pooled volume (ca 17 mL compared to 7–9 mL in the other species) and high oligosaccharide concentration. However, a number of smaller peaks (Fig. 2a) could not be characterized. Further investigation of the milk of the greater galago and other galagids, and of lorisids such as the slow and slender lorises (which contain about 7% total saccharide [25]), are needed to characterize the diversity of oligosaccharides in the Lorisiformes.

Given the purported antimicrobial and prebiotic functions of oligosaccharides, one might expect the more social diurnal/cathemeral species that maintain infant contact via carrying them (Coquerel's sifaka, mongoose lemur) to have greater diversity and amounts of milk oligosaccharides than do the noctural species that have a dispersed social system, typically forage alone, and park their young in nests or tree holes (greater galago, aye-aye) (Table 1). However, the greater galago and aye-aye had markedly different oligosaccharide patterns (Table 3), and the greater galago had as much or more total oligosaccharide in its milk than either of the lemuriformes. Thus there was no evident correlation of milk oligosaccharides to group size or reproductive pattern, despite the suggestion that this may be important in other primates [13].

We also compared the diversity of oligosaccharides in strepsirrhines to that in other primate taxa. We identified two to ten neutral oligosaccharides and two to five acidic oligosaccharides in each of the strepsirrhine milks (Table 3). By comparable methods, we found zero to three neutral and two to three acidic oligosaccharides in platyrrhine milks [4], two to five neutral and one to three acidic oligosaccharides in old world monkey (cercopithecid) milks [4], and three to six neutral and two to six acidic oligosaccharides in non-human hominoid milks [3]. This suggests that the diversity of oligosaccharides in strepsirrhine milks is comparable to that of other non-human primates; the diversity in platyrrhines may be reduced, although further species should be studied to confirm this observation.

Table 3 Comparison of oligosaccharides in the milk of strepsirrhine

Type of oligosaccharide		Species of Strepsirrhine			
		Greater Galago	Aye-aye	Coquerel's Sifaka	Mongoose Lemur
Neutral	tri		3-FL (2′-FL)[a]		3-FL[g]
				α3′-GL	α3′-GL[g]
		β6′-GL			β6′-GL[g]
	tetra		LNT[c]		
			LNnT[c]	B-tetra[f]	LNnT
		LNnT	DFL		
	penta		LNFP II[d]		
			LNFP III[d]		LNFP III
			LNFP VI[d]		
	hexa		LNnH[b,e]	LNnH	LNnH[b, h]
	hepta		FLNnH[b, e]		FLNnH[b, h]
	octa		DFLNnH[b, e]		DFLNnH[b, h]
Acidic	di			L-3′-s[f]	
	tri	3′-SL-NAc	3′-SL-NAc	3′-SL-NAc	3′-SL-NAc
		3′-SL-NGc	6′-SL-NAc	6′-SL-NAc	6′-SL-NAc
	tetra			DSL	
	penta				LSTc
	hepta			MSLNnH	MSLNnH
	octa				MSMFLNnH

[a] 2′-FL was isolated in the involuting milk sample from the aye-aye

[b] The milks of aye-aye and mongoose lemur contained LNnH and its fucosylated forms, probably FLNnH and DFLNnH (see text).

[c–h]: These oligosaccharides were characterized from fractions with a mixture of components, as described in the text.

We previously postulated that there may be a phylogenetic trend for oligosaccharide diversity to increase from platyrrhines to old world monkeys, from cercopithecid monkeys to great apes, and from great apes to humans [3, 4]. However, the strepsirrhine results presented herein, and in particular the great diversity of oligosaccharides in aye-aye and mongoose lemur milks, suggest that the phylogenetic history of oligosaccharides may be more complex than this, and the apparent lower diversity in platyrrhine milks may represent a secondary reduction. Caution is also warranted as we have only characterized the major oligosaccharides that could be isolated from the amounts of milk available to us; a greater volume of milk would be needed to identify by ^{1}H-NMR oligosaccharides present at lower concentrations. Further studies are needed with greater milk volumes, additional species, and more sensitive analytic methods to fully characterize phylogenetic patterns among primates. With a smaller set of species (and no strepsirrhines) Tao *et al*. [13] also questioned how closely oligosaccharide patterns track primate phylogeny. However, the extraordinary diversity of milk oligosaccharides found in human milk remains unique among all primates studied [1, 11]. The diversity of human milk oligosaccharides is associated with the co-presence of both type I and type II oligosaccharides.

The presence of type I and type II oligosaccharides

Oligosaccharides that contain Gal(β1-3)GlcNAc [lacto-*N*-biose I (LNB)] are considered type I oligosaccharides, whereas those that contain Gal(β1-4)GlcNAc [*N*-acetyllactosamine (LacNAc)] are type II (*e.g.*, Table 2). In human milk and colostrum, type I saccharides/oligosaccharides dominate: they are present in significantly higher concentrations than type II structures [8–10]. We detected type I saccharides (LNFP II and LNT) in aye-aye milk but not in greater galago, Coquerel's sifaka or mongoose lemur milk. Type II saccharides were also detected in aye-aye milk and these dominate over type I, in contrast to human milk. In our previous studies [3, 4], type II saccharides were detected in the platyrhines, cercopithecids and the siamang, but both type I and II saccharides were found in chimpanzee, bonobo and orangutan. However, as in the aye-aye, in these apes the concentrations of type II saccharides predominated over type I.

A recent publication [13] on milk oligosaccharides of apes (chimpanzee, gorilla, and siamang), new world monkeys (golden lion tamarin and common marmoset), and an old world monkey (rhesus macaque) lists a wide variety of oligosaccharides that were separated according to mass and retention time by MALDI and TOF mass spectrometry. Mass spectrometry has the advantage of great sensitivity and use of small sample size, but unlike ^{1}H-NMR does not elucidate the nature of chemical bonds or detailed chemical structure. "Possible identities" of these constituents were therefore assigned by reference to a human milk oligosaccharide (HMO) library. Unfortunately this may lead to misidentification of oligosaccharides, with a bias towards the type I constituents dominant in human milk. For example, by reference to the HMO library the primary oligosaccharide in rhesus macaque milk is annotated as LNT (type I) [13], but by ^{1}H-NMR we identified this oligosaccharide (using rhesus macaque milk from the same animal colony) as LNnT, the type II isomer [4], and did not detect LNT.

Our data indicate that type I oliogosaccharides are not unique to hominoids and that the ability to synthesize type I oligosaccharides predated the strepsirrhine-haplorhine divergence. Although we did not identify type I oligosaccharides in platyrrhines and cercopithecids [4] (see Fig. 1 for species), this may have been due to low concentrations in the small amounts of milk available for study by ^{1}H-NMR. Nonetheless, type I oligosaccharide concentrations are clearly elevated in hominids, and become dominant in human milk, suggesting that evolutionary factors favoring type I oligosaccharides came into play early in hominid evolution and particularly after the divergence of humans from chimpanzees 6–7 mya [15, 38]

The evolutionary advantage of a predominance of type I oligosaccharides in human milk is uncertain, but it could encourage the formation of a beneficial bifidus flora in the neonatal infant colon. A specific metabolic pathway for type I oligosaccharides has been found in bifidobacterial strains such as *Bifidobacterium bifidum* [1, 39, 40]. Of the predominant human milk oligosaccharides (HMO) in colostrum and milk, 2′-FL, LNFP I, LNDFH I and LNT [8, 41], the latter three contain LNB, *i.e.*, are type I. We hypothesize that LNB released from type I in preference of type II HMOs by *Bifidobacterium bifidum* lacto-*N*-biosidase are utilized by multple strains of colonic bifidobacteria in breast-fed infants, leading to their predominance. Albrecht *et al*. [42] compared oligosaccharides in human milk and in feces of breast-fed babies by capillary electrophoresis with a laser induced fluorescence detector coupled to a mass spectrometer (CE–LIF–MS^{n}). They found that the relative level of LNT (type I) was markedly lower in the fecal extract than in breast milk, while the relative level of LNnT (type II) was higher in the feces. This is consistent with the view that type I HMOs are more readily metabolized by the infant colonic microorganisms than are type II HMOs. If this is correct, the predominance of prebiotic type I HMOs may have had a selective advantage in terms of neonatal survival as the human lineage became highly social and therefore exposed to an increased array of pathogens [43].

Whether type I oligosaccharides (including LNFP II and LNT, as in aye-aye milk) have similar beneficial effects on

the digestive tracts of the suckling young of solitary aye-ayes or other primates is not known, but if so this may be relevant in efforts to hand-raise these species on artificial formulas [44, 45].

Other neutral oligosaccharides

The trisaccharide isoglobotriose (α3′-GL) was detected in sifaka and mongoose lemur milk, but has not been identified by ^{1}H-NMR in other primates [3, 4, 8]. Although further research is needed to identify the specific enzyme(s) involved in milk α3′-GL synthesis, the similar α-gal epitope (Gal(α1-3)Gal(β1-4)GlcNAc-R) in glycolipids and glycoproteins is synthesized in mammals by the Golgi glycosylation enzyme α1,3galactosyltransferase (α1,3GT) [46]. However, this epitope is absent in old world monkeys, apes and humans, apparently due to inactivation of α1,3GT gene in ancestral catarrhines [46]. If mammary α1,3GT is required for synthesis of α3′-GL, this trisaccharide should not be synthesized, and has not been detected, in these taxa. The Gal(α1-3)Gal unit can also be synthesized in glycolipids (but not glycoproteins) by a different enzyme (iGb3 synthase), at least in rats [47], but whether this has any evolutionary significance for milk oligosaccharide synthesis is unknown. Although we did not detect α3′-GL in the milk of greater galago, aye-aye and new world monkeys [4], we cannot exclude the possibility that α3′-GL is present in their milks at very low concentrations.

In the present study we detected the trisaccharide 2′-FL in early lactation milk from an aye-aye with a sick infant that subsequently died; it was not found in mid-lactation milk of this species. We do not know if 2′-FL is a normal constituent of aye-aye milk, or was generated by degradation of other fucosylated constituents (such as DFL) during mammary involution. 2′-FL has also been detected in chimpanzee, bonobo, gorilla and human milk or colostrum [3, 8], but not in platyrrhine or cercopithecid monkeys [4]. Tao *et al*. [13] also reported a structure with (α1-2) linked fucose in gorilla milk, but not in the other non-human primates studied (see above). In humans, 2′-FL can act as a receptor analogue that inhibits the attachment of *Campylobacter jejuni* to the infant colonic mucosa [48].

It is interesting that 3-FL (Gal(β1-4)[Fuc(α1-3)]Glc) and oligosaccharides (LNFP III, DFLNnH) containing the Lewis x epitope (Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)-R) were found in aye-aye and mongoose lemur milk. Such (α1-3) linked Fuc containing milk oligosaccharides were also detected in three species of cercopithecid monkeys and in chimpanzee, bonobo and orangutan, but not in gorilla and siamang [3, 4].

Acidic oligosaccharides

The milks of all four strepsirrhine species contained both 3′-SL and 6’-SL (Table 3). In the milk/colostrum of bonobo and orangutan [3] and monkeys [4], 3′-SL is present in greater quantities than 6′-SL. This was also the case for the milks of the strepsirrhines except the mongoose lemur (Figs. 4 and 5). This situation is the reverse of that in human milk or colostrum [41], where 6′-SL predominates over 3′-SL. A possible explanation for the dominance of 6′-SL over 3′-SL in human milk/colostrum might be the modulation of postnatal allergen-specific immune responses [49].

At least one milk oligosaccharide containing *N*-glycolylneuraminic acid (Neu5Gc), 3′-NGc-SL, is present in greater galago milk, and in the milk or colostrum of the great apes [3]. We did not detect Neu5Gc-containing oligosaccharides in siamang milk [3] or the milk and colostrum of platyrrhine and cercopithecid monkeys [4]. However, Tao *et al*. [13] reported NeuGc-containing oligosaccharides in rhesus macaques as well as in chimpanzee and gorilla, albeit in low quantity (1.1–9.4%). We may have failed to detect Neu5Gc-containing oligosaccharides in cercopithecids because of the lower sensitivity of ^{1}H-NMR spectroscopy as compared to MALDI and TOF mass spectrometry (MS) [13]. Glycoconjugates containing *N*-glycolylneuraminic acid are reportedly absent from the tissues, body fluids and milk/colostrum of healthy humans because the enzyme that converts CMP-Neu5Ac to CMP-Neu5Gc has been lost [50, 51]. Our data on hominid milks [3] are consistent with the hypothesis that this loss occurred subsequent to the divergence of humans from the ape (chimp-bonobo) lineage [52].

We identified a sulfate-containing disaccharide, 3′-*O*-lactose sulfate (L-3′-s), in Coquerel’s sifaka milk, as previously reported for the milk of the hamadryas baboon [4]. L-3′-s has also been found in the milks of the dog [29] and hamadryas baboon [4]. In humans, a sulfate-containing oligosaccharide, not a sulfate-containing disaccharide, was discovered by Guerardel *et al*. [53]. Further investigation is needed to determine if this or other sulfated saccharides occur in the milk or colostrum of other primates.

Summary

The diversity of milk oligosaccharides in some strepsirrhines (*e.g.*, aye-aye, mongoose lemur) appears to be as great as that in any other primate species, except humans. We conclude that secretion of a large spectrum of different milk oligosaccharides (including type I oligosaccharides) is likely an ancestral condition that predates the split of strepsirrhines from haplorhines. The large number of additional compounds that can be detected in primate milks by mass spectrometry [13] warrant further structural characterization by ^{1}H-NMR analysis to determine chemical identities. Further research is also needed on the milks of additional primate taxa, including lorisids, cebids, and colobines, to refine our understanding of phylogenetic patterns in milk

oligosaccharide secretion. While milk oligosaccharide patterns may not directly map the phylogeny of primates [13], it is possible that other biological factors, including developmental state at birth, type of parental care, degree of sociality and interactions with other milk components (such as additional antimicrobial constituents) may ultimately explain observed patterns. However, type of parental care and degree of sociality did not explain observed differences among strepsirrhine species. Given that oligosaccharides can be major constituents of milk (in some primate taxa, such as humans and lemurs, milk oligosaccharides exceed the amount of milk protein [25, 54]), and that their synthesis may entail considerable metabolic cost to lactating females, the widespread occurrence of milk oligosaccharides indicates the presence of selective evolutionary pressures to maintain their secretion.

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