LIPID GLOBULES AND FATTY ACIDS IN MILK OF LACTATING RACCOON (*NYCTEREUTES PROCYONOIDES*)

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In this study, milk samples (*n*=52) from nine healthy female raccoons at 3–45 days of lactation were manually collected at a regular morning hour from all active teats and used to investigate the changes of lipid globules size, fatty acids (FAs) profile and fat content.The results indicated that raccoon milk is characterized by a high fat content. Small lipid globules sizing up to 6 µm prevailed with their greatest share in milk during lactation phases I and III. The milk fat content was increasing with the proceeding lactation, whereas the content of free FAs had a decreasing tendency. Totally eighteen FAs were identified in raccoon milk. The unsaturated long-chain C18–C20 FAs were dominating (over 60%). The individual FAs contents in raccoon milk did not exceed 1%, except for palmitic, vaccenic and linoleic acids representing over 20% of the total FA content.The study results can be used for establishing the energy requirements during the suckling period for proper growth and development of puppies.

carnivora,fatty acids, milk fat, *Nyctereutes procyonoides,* raccoon milk

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INTRODUCTION

Mammals are the only group of animals with a mammary gland with the evolved mechanism of milk production. Secretion produced in the gland is the only source of food for the offspring in the first weeks after birth.In most mammals, milk is excreted only in the period of lactation and contains nutrients necessary for proper functioning of young organisms in the first days of life. In mono-estrus breeding females of Canidae, lactation takes place only once a year and this process lasts for 6–8 weeks. Malfunctioning mammary glands cause disorders in milk production and secretion which can be a persistent problem in feeding the pups. The research results of Szeleszczuk et al. (2007) indicate a current problem of mortality rate increase in fox litters. The number of still-born and dead suckling kids in the first days after parturition is significantly higher than the total of dead animals raised during the whole breeding cycle. Losses in fox offspring have still been a problem (Hansen, 2008) visible also in the other species of fur animals, both herbivorous and carnivorous. According to Szeleszczuk et al.(2008), this phenomenon can also be observed in another species belonging to carnivorous fur animals – the raccoon (*Nyctereutes procyonoides*). As indicated by Szeleszczuk et al.(2015), the perinatal period and the first week of pups' life are the most crucial for raccoon dogs. In that period, 30–48% offspring mortality has been recorded and the only food consumed by pups is the milk. Female milk is decisive directly upon puppies' proper growth and development. Milk lipids are their main energy source. Milk fat is diffused in the water phase of milk in the form of small lipid globules stabilised due to their coating (El-Zeini, 2006). The globules diameter is 0.1–15 µm. They can

be classified into three size categories: small (≤6 μm), medium (7–10 μm) and large (≥10 μm). Milk fat of mammals consists of simple and complex lipids, free fatty acids (FAs) and accompanying substances. The simple lipids – triglycerides – have the biggest share in the total milk fat (96–99%). They are esters composed of three molecules of FAs and one molecule of glycerol (Jensen et al., 1991).

FAs are one of the most important substances determining the development of both humans and animals in prenatal and postnatal periods. These compounds are responsible for cell energetic processes and represent the main building element of cytomembranes (Bobinski, Bobinska, 2020).

One of the factors affecting milk lipids is the lactation stage (Mesilati-Stahy, Argov-Argaman, 2014). During lactation, significant changes can be observed in milkfat and the FA content. Studies in different mammals as well as humans have been conducted (Jiang, 2020). However, little information on lipids in Canidae milk and its changes during lactation (Szeleszczuk et al.,2017) has been available in the subject literature to date. Therefore the purpose of the study was to determine the fat content, the size and number of lipid globules, and the FAs profile inr accoon (*Nyctereutes procyonoides*) milk at various stages of lactation.

MATERIAL AND METHODS

Animals

Animals were handled following the guidelines for animal experiments set in the Directive 2010/63/EU regarding the protection of animals used for experiments, and in accord with the permission from the II Local Ethics Committee in Krakow No. 17/2010 and No. 1/2020.

Nine lactating female raccoons, 2–3 years old,were used in this study. Before the reproductive season, females were subjected to health selection and preventive procedures (deworming and protective vaccinations). The animals were fed in accordance with the feeding standards recommended for particular lactation periods for this species (NRC, 1982) and fresh water was available *ad libitum*. The females in lactation were fed three times daily. Each female nursed on average 6 puppies.

Milk sampling

The lactation period was divided into three phases: phase I (early): up to 15 days; phase II (middle): from 16 to 30 days; and phase III (later): from 31 to 45 days.

Totally 52 milk samples (phase I, $n = 16$; phase II, $n = 18$; phase III, $n = 18$) were collected in days 3–45 of lactation at weekly intervals. The samples (10–32 ml) were collected from all active milk glands of the females during a diagnostic examination by a veterinarian.The milk samples were taken at regular hours before early morning feeding. Before milking, the milk gland health condition was examined. Its consistency, soreness or temperature was inspected manually. Blood or pus secretion was checked at each teat. Milk intended for analysis was collected exclusivelyf rom healthy raccoon females with healthy milk glands. Each female was subcutaneously injected at the scruff of the neck with 0.2 IU of oxytocin (*Oxytocinum* Biowet, Poland) using a 2.54 cm 25-gauge needle. After 10 min to allow the hormones work, the teats were massaged.Milk for the assay was collected manually from each active teat to 40 ml sterile plastic containers and 2 ml Eppendorf tubes. The milk glands were tried to be emptied completely to obtain the identical volume of milk as during the pups feeding.For the fat analysis,the collected milk was preserved by Broad Spectrum Microtabs II (dispenser with 800 tablets) (Advanced Instruments, USA) (Barlowska, 2007). Milk samples were transported to the laboratory in a cooled bag and stored deep frozen(–20°C) until analyses.

Laboratory analyses

The fat and free FAs contents in raccoon milk samples were determined using a MilkoScan FT2 analyser (FOSS, Denmark) calibrated by laboratory using cow milk samples. The FAs profile was determined according to Folch et al.(1957) using a gas chromatograph Varian 450-GC with a flame ionization detector (FID) and a CP-SIL 88 column (length 100 m, diameter 0.25 mm) for FAME (all Agilent Technologies, USA). The injector temperature was 220°C and the detector temperature 250°C. The carrier gas was helium (1 ml min^{-1}).The milk samples were extracted using chloroform and methanol mixed 2:1. Then, 0.58% NaCl was added, the samples were centrifuged, 1 cm^3 of the bottom layer was collected and the solvent was evaporated under nitrogen (at 50°C). Totally 0.5M NaOH in methanol, 10% BF_3 in methanol and heptane were added respectively to the dry residue. After adding each reagent, the test tube was screwed and put into a water bath (73°C). Then, a saturated solution of NaCl was added. After separating the layers, the heptanoic layer was removed and placed in a test tube containing anhydrous Na_2SO_4. Individual FA methyl esters were identified by comparison to the standard mixture (Supelco 37 component FAME Mix; Sigma-Aldrich Co., USA). The FA content was expressed as a percentage of the total identified FAs.

The size and amount of lipid globules were determined in the samples of unpreserved raccoon milk according to the method worked out and later modified by Barlowska (2007). The performed slides were examined under a microscope Nikon ECLIPSE

Table 1.Quantity (ml) and proportion of lipid globules (%) of raccoon milk depending on lactation phase

Items	Phase of lactation		
	I	II	III
Milk quantity (ml)	7.00[a] (7.00–10.00)	22.00[b] (17.00–27.00)	17.00[b] (7.00–22.00)
Lipid globules proportion (%)			
< 6 µm	85.34[a] (84.77–89.73)	75.00[a] (68.75–84.28)	83.46[a] (81.45–93.48)
7–10µm	13.61[a] (9.73–15.23)	15.24[a] (13.27–25.00)	12.40[a] (6.05–14.97)
> 10µm	0.54[a] (0.39–1.05)	0.48[a] (0–1.56)	2.97[a] (0.47–4.30)

values are presented as median and interquartile range (Q_1= lower quartile, Q_3= upper quartile)

[a,b]within a row, means with a common superscript do not differ significantly (P <0.05)

Table 2.Effect of lactation phase on the lipid content of raccoon milk

Items	Phase of lactation		
	I	II	III
Fat (%)	9.25[a] ± 1.88	11.42[b] ± 3.43	13.61[c] ± 1.69
Free fatty acids (%)	4.15[a] ± 0.06	1.18[b] ± 0.41	1.79[c] ± 0.46

values are presented as mean ± SD

[a–c]within a row, means with a common superscript do not differ significantly (P <0.05)

E 600 (Nikon, Japan) at 40× magnification. The amount and size of lipid globules were measured using a MULTISCAN programme, classifying the globules to three size categories: <6 µm, 7–10 µm, and > 10 µm.

Statistical analysis

One-way analysis of variance (ANOVA) for repeated measures was performed (Statistical Analysis System, 2014, Version 9.4). The significance of differences between the mean values of the tested groups was determined by the Tukey test at P<0.05. For non-normally distributed variables, the non-parametric Friedman test and post-hoc Nemenyi test were used (R Core Team,2013).

RESULTS

The amount of milk collected from the lactating female raccoons was small in comparison to that produced by other mammals.The highest milk production was noted in lactation phase II, the median value was higher by about 32% than in phase I (Table 1). In lactation phase III, the amount of collected milk was lower if compared to phase II.

Small lipid globules sizing up to 6 µm were prevailing in the raccoon milk smear, attaining the biggest share in lactation phases I and III (similar medians were observed– 85.34 and 83.46, respectively). However, the proportion of lipid globules over 10 µm was the lowest throughout all lactation phases (Table1).

The raccoon milk showed a high fat content. The milk fat content significantly (P< 0.05) increased with the proceeding lactation. Contrarily, the free FAs proportion was significantly decreasing – from 4.15% in lactation phase I to 1.79% in phase III(Table 2).

Only eighteen FAs were detected in the tested milk samples. Most of these FAs were present in small amounts, forming less than 1% of total FA content. Conjugated linoleic acid (CLA),showing significant biological properties, was also present. Three fatty acids – C16:0 (palmitic acid), C18:1 n7*trans* (vaccenic acid), C18:2 n6 (linoleic acid) – constituted over 20 % of the total FA content in raccoon milk (Table 3).

Results in Tables 4, 5 reveal a clear relationship between the particular FAs contents in raccoon milk and the lactation phases. The FAs profile was changing significantly (P<0.05) in relation to the lactation phase. With the proceeding lactation, a special increase was noted in unsaturated FAs C16:1 n7 (palmitoleic acid) and C18:2 n6 (linoleic acid), whereas the contents of saturated FAs C6:0 (caproic acid), C12:0 (lauric acid), C14:0 (myristic acid), C15:0 (pentadecanoic acid) and unsaturated FAsC18:1 n9 *cis* (oleic acid), C20:1 n9 (eicosenoic acid), C20:2 n6 (eicosadienoic acid) were decreasing.The raccoon milk analysed showed a predominance of unsaturated FAs.

DISCUSSION

The information on the raccoon milk lipid contentis missing in available literature. Therefore, the present research results were compared with results available on other carnivores such as Canidae and Felidae.

Table 3.Fatty acids profilein raccoon milk (%of total fatty acids)

Fatty acids		Percentage of total FA
C4:0	butyric	0.05 ± 0.02
C6:0	caproic	0.25 ± 0.11
C8:0	caprylic	0.01 ± 0.00
C10:0	capric	0.04 ± 0.02
C12:0	lauric	0.52 ± 0.36
C14:0	myristic	1.67 ± 0.31
C15:0	pentadecanoic	0.13 ± 0.04
C16:0	palmitic	23.93 ± 1.11
C16:1n7	palmitoleic	4.67 ± 0.44
C18:0	stearic	4.99 ± 0.49
C18:1n9 cis	oleic	0.20 ± 0.10
C18:1n7 trans	vaccenic	38.13 ± 2.28
C18:2n6	linoleic	20.27 ± 1.48
C18:2 CLA	conjugated linoleic acid	0.08 ± 0.09
C18:3n3	linolenic	1.60 ± 0.35
C20:0	arachidic	0.05 ± 0.01
C20:1n9	eicosenoic	0.38 ± 0.17
C20:2n6	eicosadienoic	0.25 ± 0.02
Remaining acids (altogether)		3.05

values are presented as mean ± SD

In our experiment, the amount of milk collected from the female raccoons (2–32 ml per dam) was lower in comparison to that yielded by other farm animals. Foxes, belonging also to the family Canidae,yielded 2–300 ml milk per dam per day 2–3 days post partum, and the yield per dam increased to 7–800 ml in days 13–16 post partum. The maximum daily milk yield observed in a silver fox dam nursing eight 16-day-old cubs was estimated at 858 ml (Ahlstrom, Wamberg, 2000). It is not easy to compare data from different studies including the milk yield data, because many of the results are influenced by species, animal feeding and stage of lactation.

In the raccoon milks mear performed within this study, small lipid globules sizing up to 6 µm prevailed while globules over 10 µm were less frequent. Similar trends were also observed in silver foxes (Szeleszczuk et al.,2017). According to Singh (2019) the occurrence of smaller globules is more beneficial as they have a larger surface for a contact with enzymes that decompose fat, whereby they are more easily digestible for the organism. Similarly, Martini et al. (2012) stated that smaller lipid globules are more available and susceptible to lipolytic processes. Moreover, small lipid globules contain more short-chain FAs and less long-chain ones as opposed to greater globules. Lu et al. (2016) found that fat globules show species variability. Many studies on fat globules in ruminant milks have been conducted, unfortunately, there is little information about fat globules in carnivore milk in scientific literature, making the comparison difficult.

In our study the detected fat content in raccoon milk was high (9.25–13.61%). Similar results have been reported for the milk of silver and blue fox females

Table 4.Saturated fatty acids profile (%) of raccoon milk in relation to lactation phase

Saturated fatty acids I		Phase of lactation		
		II	III	
Short chain FA	C4:0	0.06^{a} ± 0.01	0.04^{a} ± 0.02	0.05^{a} ± 0.01
	C6:0	0.45^{a} ± 0.05	0.30^{b} ± 0.16	0.22^{b} ± 0.04
	C8:0	0.02^{a} ± 0.01	0.01^{b} ± 0.00	0.01^{b} ± 0.01
	C10:0	0.06^{a} ± 0.03	0.04^{ab} ± 0.02	0.04^{b} ± 0.02
	total	0.53	0.35	0.37
Long chain FA	C12:0	0.85^{a} ± 0.02	0.86^{a} ± 0.24	0.24^{c} ± 0.01
	C14:0	2.49^{a} ± 0.41	1.93^{b} ± 0.28	1.46^{c} ± 0.12
	C15:0	0.18^{a} ± 0.04	0.13^{a} ± 0.05	0.12^{a} ± 0.04
	C16:0	24.20^{a} ± 0.84	25.03^{a} ± 0.29	23.00^{b} ± 0.39
	C18:0	4.97^{ab} ± 0.22	5.34^{a} ± 0.54	4.70^{b} ± 0.14
	C20:0	0.04^{ab} ± 0.01	0.04^{a} ± 0.01	0.05^{b} ± 0.01
	total	32.73	33.33	29.57

values are presented as mean ± SD

$^{a–c}$within a row, means with a common superscript do not differ significantly (P <0.05)

Table 5. Unsaturated fatty acids profile (%) of raccoon milk in relation to lactation phase

Unsaturated fatty acids I		Phase of lactation		
		II	III	
MUFA	C16:1n7	$4.27^{a} \pm 0.43$	$4.42^{a} \pm 0.41$	$4.88^{a} \pm 0.37$
	C18:1n9*cis*	$0.33^{a} \pm 0.15$	$0.23^{ab} \pm 0.12$	$0.17^{b} \pm 0.08$
	C18:1n7*trans*	$38.48^{ab} \pm 0.53$	$36.59^{a} \pm 2.63$	$39.42^{b} \pm 0.70$
	C20:1n9	$0.72^{a} \pm 0.27$	$0.52^{b} \pm 0.07$	$0.25^{c} \pm 0.11$
	total	43.80	41.76	44.72
PUFA	C18:2n6	$18.00^{a} \pm 0.64$	$18.82^{a} \pm 0.64$	$21.48^{b} \pm 0.46$
	C18:2 CLA	$0.04^{a} \pm 0.03$	$0.13^{b} \pm 0.11$	$0.03^{a} \pm 0.02$
	C18:3n3	$1.50^{a} \pm 0.50$	$1.32^{a} \pm 0.14$	$1.84^{a} \pm 0.29$
	C20:2n6	$0.34^{a} \pm 0.04$	$0.27^{b} \pm 0.02$	$0.24^{b} \pm 0.01$
	total	19.88	20.54	23.59

MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids

values are presented as mean ± SD

$^{a–c}$within a row, means with a common superscript do not differ significantly ($P < 0.05$)

showing an average fat content 10.23% and 11.16%, respectively (Szeleszczuk et al., 2016). In domestic dog milk, lipid concentration was ranging from 11.25 % to 13.72% (Adkins et al., 2001). Osthoff et al. (2007) reported that serval milk contained 152.6 ± 62.3 g fat per kg milk during mid-lactation**.**For suckling rabbits, an average lipid content was 12.9 g per 100 g milk, so it is clear that the fat component is quantitatively the greatest energy source (Maertens et al., 2006).In raccoon milk,the lipid content increased with the proceeding lactation. Oftedal (1984) recorded that dog milk contained on average 9.47% fat, and milk fat content decreased from 10.9% (days 7–9) to 8.7% (days 22–23) and then increased to 9.16% (days 29–30). According to Jacobsen et al.(2004), in cat milk,the fat percentage decreased slightly by the 3rd week of lactation and gradually increased until the 6th week when it got slightly above the initial level. In contrast, Lonnerdal et al.(1991) indicated that the fat concentration in Beagle dog milk increased from 2.4% to 4.5% in early lactation and then decreased to 2.7% in late lactation. For German Shepherds, Dokoupilova et al. (2016) reported a fat content increasing since the 10th till the 25th day of lactation. Similar tendencies were observed by Adkins et al. (1997) in cat milk. Its fat concentration increased significantly during lactation from 1.4% to about 5% in the later stage.

The profile of FAs identified in raccoon milk is not as wide as in ruminant milk. Only eighteen FAs have been detected in our milk samples.The FAs levels in milk changed with lactation phases. The unsaturated long-chain C18-C20 (over 60%)FAs were dominating in raccoon milk. Similar results were also noted in silver foxes (Szeleszczuk et al., 2017). However, according to Maertens et al. (2006), the FAs profile in rabbit milk was characterized by a very high content of short-chain FAs due to the high (50%) content of C8:0-C12:0 FAs. According to these authors, as many as 70% of the milk FAs were saturated (SFA), whereas the percentage of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids was 13% and 16%, respectively. Osthoff et al. (2007) reported that serval milk contains up to 313.3 g saturated FAs per kg of milk fat. In our study, as many as 61% of all FAs determined in raccoon milk were present in small amounts, below 1%. CLA, the FA with significant biological properties, was also present. The acids such as C16:0 (palmitic acid), C18:1n7 *trans* (vaccenic acid), and C18:2n6 (linoleic acid) represented over 20 % of the total FA content in raccoon milk.The FAs compositionin milk fat from cheetah showed the same general pattern as in carnivores. Milk fat was rich in C16:0 (palmitic), C18:1 (oleic), and C18:2 (linoleic) acids (Osthoff et al., 2006).

Several studies have demonstrated that the share of particular FAs depends on the size of lipid globules. Small lipid globules contain a higher proportion of unsaturated FAs and less short-chain FAsand stearic acid in relation to globules classified as medium and large sized (Michalski et al., 2001; Wiking et al., 2004; Couvreur, Hurtaud, 2017). Similar trends have also been observed in our study.

Quantitative and qualitative differences in the milk fat FAs content of mammals are influenced mainly by feeding and the construction of the alimentary canal of each animal species. Like all the members of the family Canidae,raccoons belong to carnivorous animals. Their alimentary canal is very short in comparison to ruminants. Vegetable roughages are not used in

feeding domestic and farm Canidae, the basis of their feeding is meat and slaughterhouse offal. According to Contarini et al.(2009), SFAs are a valuable component of animal lipids that is not present in vegetable fats. The principal dietary sources of CLA for domestic and farming Carnivores are animal products. In Canidae, SFAs are absorbed from the alimentary canal without creating chylomicrons, so they do not contribute to a blood lipid level increase.

CONCLUSION

This study has demonstrated that raccoon milk is characterized by the high fat content. Small lipid globules were prevalent in the milk achieving the biggest share in phases I and III of lactation. The fat content was increasing with the proceeding lactation, whereas the content of free FAs was decreasing. Totally eighteen FAs were identified in raccoon milk. The content of most of them did not exceed 1%, except for palmitic, vaccenic and linoleic acids, which made up over 20 % of the total FA content in raccoon milk. Milk lipids are the main energy source and the resent results can be used to determine the energy requirements during the suckling period for proper growth and developmentof raccoon puppies.

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