De novo lipogenesis is suppressed during fasting but upregulated at population decline in cyclic voles

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Abstract

Arvicolines are susceptible to the development of fatty liver during short-term fasting. We examined the potential role of *de novo* lipogenesis (DNL) (i) in the development of fasting-induced fatty liver and (ii) during a population cycle by measuring the mRNA expression of acetyl-CoA carboxylase-1 (ACC1) and fatty acid synthase (FAS). Laboratory voles (*Microtus oeconomus* and *Microtus arvalis*) were fed or fasted for 12 or 18 h and their liver mRNA levels were determined. Both species showed decreased mRNA expression of ACC1 and FAS during fasting. This suggests that DNL does not participate in the development of fatty liver in voles, different from human non-alcoholic fatty liver disease. In wild bank voles (*Myodes glareolus*), the mRNA levels of the genes of interest were higher during the population decline compared to the increase phase. In conclusion, DNL was suppressed during acute fasting but upregulated during a long-term population decline—a period of purported scarcity of high-quality food.

Keywords: Acetyl-CoA carboxylase-1, *de novo* lipogenesis, fatty acid synthase, population cycles

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Introduction

Voles (Arvicolinae) are interesting models to study fatty liver due to their peculiar physiology. Voles require nutrition every few hours and have inadequate fasting responses.^{1–3} They exhibit increased hepatic triacylglycerol (TAG) content after 4–18 h of fasting.^{2,4} Despite the different etiologies (food deprivation *vs.* human obesity), the lipid accumulation in voles shares similarities with that of human non-alcoholic fatty liver disease (NAFLD).^{2,5} A primary cause for both seems to be the high influx of non-esterified fatty acids (NEFA) into the liver due to lipid mobilization (fasting^{2,4}) or as an aspect of the metabolic syndrome (obesity⁶). This can overwhelm the ability of the liver to utilize the fatty acids (FA) for energy production or to secrete them in very-low-density lipoprotein (VLDL).⁷ In addition to macrovesicular steatosis, the vole fatty liver has FA manifestations similar to NAFLD, including decreased hepatic proportions of long-chain polyunsaturated fatty acids (PUFA), especially n-3 PUFA.^{4,5} This has important ramifications, as the depletion of n-3 PUFA can shift the balance from TAG hydrolysis to FA synthesis and reduce VLDL secretion, thus enhancing lipid accumulation.

In the American mink (*Neovison vison*), *de novo* lipogenesis (DNL) increases during food deprivation simultaneously with the development of fatty liver.⁷ The hepatic TAG load is also partly aggravated by DNL in human NAFLD,⁶ but the role of DNL in fatty liver of voles remains unknown.

In addition to being susceptible to acute fasting,¹ arvicoline populations in northern Europe display seasonal and multiannual (3–5 years) fluctuations,^{8,9} which may predispose voles to prolonged periods of food shortage and/or low-quality food. The densities are the highest in late autumn–early winter with a decline towards the end of winter.¹⁰ Food scarcity was proposed as the factor, which limits population growth in the peak phase and initiates the decline, while mortality by predation accelerates and prolongs the decline.^{9,11,12} It has been assumed that voles would be malnourished and in a poorer physiological condition in declining populations^{10,13} predisposing them to infections and parasites further reducing their condition.¹⁴

We assessed (i) the possible role of DNL in the fasting-induced fatty liver of voles and (ii) its significance in wild voles susceptible to seasonal fluctuations in food availability and displaying cyclic population dynamics. It was hypothesized that, in contrast to NAFLD patients⁶ and

carnivorous mink,⁷ DNL would not exacerbate steatosis during short-term fasting in voles. For wild voles, we hypothesized that prolonged exposure to food of lesser quantity and/or quality (fat content) after the population peak would increase DNL to meet the lipid requirements.

Materials and methods

The experiments on the laboratory voles were approved by the National Animal Experiment Board (ESLH-2007-06169/Ym-23). The tundra voles (*Microtus oeconomus*; 15 males, 15 females) and common voles (*Microtus arvalis*; 15 males, 15 females) derived from the colonies of the University of Eastern Finland.⁴ Wild overwintered bank voles (*Myodes glareolus*; 16 males, 12 females) were live-trapped around the Konnevesi Research Station (62.616°N, 26.349°E) during the increase ($N=16$; May 2008) and decline phases ($N=12$; May 2009) of their population cycle.¹⁵ According to the Finnish Act on the Use of Animals for Experimental Purposes (62/2006) and a further decision by the National Animal Experiment Board (16 May 2007), live-trapping is not an animal experiment. No permit was required to euthanize and subsequently sample the bank voles. The tundra and common voles were divided into three experimental groups (5 males, 5 females) per species: 1) fed control group, 2) group fasted for 12 h and 3) group fasted for 18 h before sampling. At the end of the experiments, liver samples were dissected after euthanasia with diethyl ether, processed for histology or frozen in liquid nitrogen and

stored at -80°C .⁴ The bank voles were euthanized and sampled similarly after 1 h of fasting.¹⁵

For the molecular biology assays,⁷ liver total RNA was extracted with the RNeasy kit (Qiagen, Germantown, MD) and cDNA synthesized by using the MultiScribe™ RT with random primers (High-Capacity complementary DNA Reverse Transcription Kit with RNase Inhibitor, Applied Biosystems, Foster City, CA). For the target genes, species-specific products were generated with primers designed on previously published sequences (acetyl-CoA carboxylase-1 [ACC1] and fatty acid synthase [FAS]: *Mus musculus*; hypoxanthine phosphoribosyltransferase 1 [HPRT1]: *Cricetulus* sp.), sequenced and submitted to GenBank (Table 1). mRNA was analyzed by real-time quantitative PCR (RT-qPCR) with the Lightcycler® 480 II (Roche Applied Science, Indianapolis, IN) using primers based on *M. arvalis* sequences (Table 1). The samples were analyzed in triplicates with the acceptable coefficient of variation <10%. All RT-qPCR were run on 1 µL of a 1:10 dilution of sample cDNA, in a reaction mix consisting of 1X GoTaq® Flexi Buffer, 2.5 mmol/L MgCl₂, 0.2 mmol/L dNTP mix (each), 0.8 U GoTaq® Hot Start DNA Polymerase (Promega, Fitchburg, WI), 1X EvaGreen® (Biotium, Hayward, CA), and 400 nmol/L each specific forward and reverse primer (Table 1) in a reaction volume of 10 µL. The specific reaction conditions are presented in Table 2.

For the Western blotting, subsamples of liver were homogenized with a bead mill (Micro Smash MS-100, TOMY

Table 1 The primers used for generating species-specific sequences of the target genes, and the primer information and GenBank accession # for the target genes of the voles

Primer sequences		Accession #	Location on sequence	Product length (bp)
FAS				
Forward	5'-TTTCTGCCATCTCCAAGACC-3'	NM_007988 ^a	6124–6143	447
Reverse	5'-CAGAGGAGAAGGCCACAAAG-3'		6570–6551	
ACC1				
Forward	5'-ACATGAAGGCTGTGGTGATG-3'	NM_133360 ^a	2968–2987	652
Reverse	5'-CTGTTTAGCGTGGGGATGTT-3'		3619–3600	
HPRT1				
Forward	5'-CCAGCGTCGTGATTAGTGAT-3'	J00060 ^b	107–126	564
Reverse	5'-CAAGGGCATATCCAACAACA-3'		670–651	
		Location on sequence ^d	Product length (bp)	Accession #
FAS				
Forward	5'-TTGTGCTAACTTCCCGGTCT-3'	93–112	108	FJ944554 ^c
Reverse	5'-AGCTGGCATTGCTTGTTG-3'	200–183		FJ944553 ^d GQ844290 ^e
ACC1				
Forward	5'-ATTGATCAGTTGTGTGGACGGG-3'	134–155	77	FJ944552 ^c
Reverse	5'-CTGAGTTGAGTTAGGTCTGTGAGG-3'	210–187		FJ944551 ^d GQ844291 ^e
HPRT1				
Forward	5'-CTGGAAAGAATGTCTTGATTGTTG-3'	326–349	93	JN701898 ^c
Reverse	5'-TTTTGGGGTTGTACTGCTTG-3'	418–399		GU645977 ^d JN701897 ^e

FAS: fatty acid synthase, ACC1: acetyl-CoA carboxylase-1, HPRT1: hypoxanthine phosphoribosyltransferase 1, bp: base pair.

^a*Mus musculus*, ^b*Cricetulus* sp., ^c*M. oeconomus*, ^d*M. arvalis*, ^e*M. glareolus*.

Table 2 Reaction conditions for the real-time quantitative PCR analyses

Target gene	FAS	ACC1	HPRT1
PCR cycles (N)	45	40	40
Preamplification (°C, hold time, ramp rate)	95°C, 2 min, 4.8°C/s	95°C, 2 min, 4.8°C/s	95°C, 2 min, 4.8°C/s
Denaturation (°C, hold time, ramp rate)	95°C, 30 s, 4.8°C/s	95°C, 5 s, 4.8°C/s	95°C, 30 s, 4.8°C/s
Annealing (°C, hold time, ramp rate)	61°C, 30 s, 2.5°C/s	65°C, 10 s, 4.8°C/s	60°C, 30 s, 2.5°C/s
Extension (°C, hold time, ramp rate)	72°C, 30 s, 4.8°C/s	72°C, 5 s, 4.8°C/s	72°C, 30 s, 4.8°C/s
Melt curve (°C, hold time, ramp rate)	95°C, 5 s, 4.8°C/s	95°C, 5 s, 4.8°C/s	95°C, 5 s, 4.8°C/s
	65°C, 20 s, 2.5°C/s	65°C, 20 s, 2.5°C/s	65°C, 20 s, 2.5°C/s
	70°C, 1 s, 4.8°C/s	70°C, 1 s, 4.8°C/s	70°C, 1 s, 4.8°C/s
	70–95°C, 2 min, 0.11°C/s	70–95°C, 2 min, 0.11°C/s	70–95°C, 2 min, 0.11°C/s

FAS: fatty acid synthase, ACC1: acetyl-CoA carboxylase-1, HPRT1: hypoxanthine phosphoribosyltransferase 1.

Digital Biology, Tokyo, Japan) in 10 volumes of ice-cold RIPA buffer (50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1% Tergitol (NP-40), 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors. The homogenates were centrifuged at 13,000 rpm for 15 min at 4°C, and the protein concentrations estimated using the PierceTM BCA assay (Thermo Fisher Scientific, Hudson, NH). The protein extracts were heated to 95°C for 5 min in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing β -mercaptoethanol, and 5 μ g was loaded onto each lane of a 4–15% acrylamide gel (Mini-PROTEAN[®] TGXTM, Bio-Rad Laboratories). The gels were visualized upon UV activation to determine the relative total protein on each lane (ChemiDoc, Bio-Rad Laboratories), then the protein was transferred to a polyvinylidene fluoride membrane. After washing with phosphate-buffered saline-Tween (PBS-T; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 2 mmol/L KH₂PO₄, 0.05% Tween 20), and blocking in PBS-T with 5% BSA for 60 min at room temperature, the membrane was incubated for 60 min with a 1:3000 dilution of rabbit polyclonal anti-FAS in blocking buffer (ab22759: Abcam, Cambridge), rinsed five times in PBS-T at room temperature, incubated with a 1:30,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories), visualized with ClarityTM Western ECL substrate and imaged using the ChemiDoc system. The proteins were quantified using the Image LabTM software (Bio-Rad Laboratories). All values were normalized to the signal of a pooled sample loaded onto each gel. Due to the small amount of samples remaining, it was only possible to conduct the FAS protein analysis with Western blotting.

The plasma NEFA levels (bank voles), liver fat-%, TAG contents, histology, and the relative proportions (mol-%) of hepatic FA were reported previously.^{4,15} Multiple comparisons were performed with the Kruskal-Wallis nonparametric analysis of variance and the Dunn's *post hoc* test, and comparisons between two groups with the Mann-Whitney *U* test (SPSS v19 program, IBM, Armonk, NY). Correlations were calculated with the Spearman correlation coefficient (r_s). The *P* value < 0.05 was considered significant. The results are presented as the mean $\pm$ SE.

Results

In the tundra voles, the decrease in the mRNA levels of both target genes was significant at 12 h of fasting (Kruskal-Wallis test, *P* < 0.001) and in the common voles at 18 h (Kruskal-Wallis test, *P* < 0.01; Figure 1). The FAS protein expression decreased at 12 h in the tundra voles and remained low at 18 h (control: 81.6 ± 43.03 , 12 h: 0.2 ± 0.06 , 18 h: 0.2 ± 0.05 arb. unit; Kruskal-Wallis test, *P* = 0.001), but in the common voles, there were no significant differences (2.1 ± 0.72 , 2.3 ± 1.05 and 1.2 ± 0.29 arb. unit). The bank voles of the decline phase had higher mRNA expressions of ACC1 and FAS (Mann-Whitney *U* test, *P* < 0.05; Figure 1).

ACC1 and FAS mRNA levels correlated negatively with liver fat-% and plasma NEFA concentrations, but positively with liver 18:0 proportions in the laboratory voles (Table 3). A consistent negative correlation was also present regarding ACC1 and FAS *versus* 16:1n-7. In the wild voles, FAS mRNA levels correlated with liver 16:0 and 18:1n-9. Selected n-3 PUFA (20:5n-3 and 22:6n-3) showed positive correlations with the target gene mRNA in the laboratory voles.

Fasting increased the histological scores for macro- and microvesicular steatosis in the laboratory voles.⁴ None of the bank voles displayed steatosis.¹⁵ In the tundra voles, FAS mRNA correlated inversely with macro- and microvesicular steatosis ($r_s = -0.600/-0.665$, *P* < 0.01) and the same was observed for ACC1 ($r_s = -0.705/-0.790$, *P* < 0.01). The correlations were not significant in the common voles except for ACC1 and microvesicular steatosis ($r_s = -0.436$, *P* < 0.05).

Discussion

We were able to confirm the hypothesis of no DNL participation in vole steatosis evidenced by the decreased ACC1 and FAS mRNA levels in the fasted common voles at 18 h and in the tundra voles at 12 h, with further support from the Western blotting data with the caveat that mRNA level changes are not always reflected at the protein level.¹⁶ The negative correlations between the mRNA and the liver TAG concentrations/histological scores of steatosis were more significant in the tundra than in the common voles. The reason for this remains unknown, but previous studies

Figure 1 Effects of fasting on the liver *de novo* lipogenesis-related mRNA expression (gene of interest/housekeeping gene + SE) in the common voles (*M. arv*) and tundra voles (*M. oec*) and of population phase on the mRNA expression in the bank voles (*My. gla*). ACC1: acetyl-CoA carboxylase-1, FAS: fatty acid synthase, HPRT1: hypoxanthine phosphoribosyltransferase 1.

*Significant differences between a fasted group and the corresponding control group or between the different population cycle phases (Kruskal–Wallis test, Mann–Whitney *U* test, $P < 0.05$)

indicate that fasting tundra voles exhibit a more profound depletion of liver glycogen stores, which could lead to potentially earlier dependence on fat mobilization² and, thus, more rapid development of fatty liver.⁴ Some correlations between ACC1/FAS mRNA and particular FA may not have been causal but confounded by the mobilization of lipids from adipose tissue to be transferred into liver as NEFA and by the decreases that occurred in hepatic proportions of some FA due to fasting.^{4,5}

As it is known that PUFA can inhibit the induction of ACC and FAS in rats,¹⁷ the elevated NEFA available for vole livers could have affected the rate of DNL. Although not analyzed in the present study, one possible path of transmission could be via the suppression of sterol regulatory element-binding protein 1 by 20:5n-3 and 22:6n-3.¹⁸ However, as these PUFA decrease in vole livers during fasting,^{4,5} similar to FA manifestations of NAFLD patients,¹⁹ this hypothesis seems implausible. A more convincing explanation for the suppression of DNL derives from the observations that fasted voles also experience a depletion of liver glycogen stores^{1,2,4} with consequent hypoglycemia¹ and a lack of carbohydrate-based substrates (i.e. glucose²⁰) for DNL. Glucose is also required in the pentose phosphate pathway for the generation of NADPH, the hydrogen donor for DNL.²¹ Furthermore, DNL results in the formation of malonyl-CoA, which regulates the entry of FA into mitochondria. It is perhaps critical for the voles that DNL would not be upregulated during severe negative energy balance, as this could also suppress FA oxidation.

When vole steatosis is compared to human NAFLD, there are several important similarities. While the etiologies are dissimilar, the primary mechanism of fat accumulation in both seems to be the increased influx of NEFA into the liver.^{2,4,6} The macrovesicular steatosis and FA manifestations (e.g. depletion of long-chain n-3 PUFA) are also shared by both.^{4,5,19} DNL exacerbates steatosis in humans⁶ but not in voles, which may be related to the different availability of carbohydrate-based substrates.^{1,2,4,22} Another major difference is insulin sensitivity: when hepatic TAG accumulate in lean fasted animals, they do not cause hepatic insulin resistance that is a characteristic of NAFLD patients.²³ While the vole model is not fully comparable to NAFLD, its benefits are the very rapid accumulation of TAG and the modification of hepatic FA profile independent of DNL. This could enable us to isolate the NEFA influx component of steatosis and to use voles as a model to investigate this part of the pathogenesis of fatty liver further.

The wild voles experienced a clear increase in DNL-related gene expression during the decline phase with no increase in the liver fat-%. The higher DNL could represent a long-term regulatory mechanism of increased endogenous lipid synthesis for the voles maturing during food scarcity and/or exposure to low-quality food. There are no detailed studies on the wild vole diet in terms of lipid content and composition during the phases of their population cycles. Hence, it cannot be ascertained that food lipids would vary as a function of population density. A more comprehensive explanation would be variations in the quantity of preferred food. The voles in the spring of the decline year had survived a winter, during which the high-density populations had presumably consumed most of the preferred food, leaving only lower quality items for consumption in early spring until the snow has melted. In contrast, the voles sampled in the spring of the peak year had not endured strong competition for resources, and high-quality food items were more likely to have remained abundant until spring. Thus, a density-dependent decrease in the availability of high-quality food could, together with

Table 3 Spearman correlation coefficients between the mRNA levels of the target genes/hypoxanthine phosphoribosyltransferase 1 and selected variables of morphology and biochemistry of the voles

	<i>M. oeconomus</i>		<i>M. arvalis</i>		<i>M. glareolus</i>	
	FAS	ACC1	FAS	ACC1	FAS	ACC1
Liver fat-%	−0.548**	−0.658**	−0.797**	−0.796**	−0.040	−0.123
Liver TAG	−0.668**	−0.625**	−0.377	−0.477*	0.060	0.169
WAT	0.037	−0.247	−0.295	−0.240	−0.541**	−0.492**
WAT % BM	−0.030	−0.286	−0.415*	−0.356	−0.500**	−0.445**
Plasma NEFA	−0.596**	−0.830**	−0.532*	−0.523*	−0.025	0.219
Liver 16:0	0.487*	0.023	0.106	0.064	0.497**	0.349
Liver 18:0	0.455*	0.730**	0.607**	0.672**	−0.209	−0.115
Liver 16:1n-7	−0.428*	−0.753**	−0.569**	−0.636**	0.224	0.254
Liver 18:1n-9	−0.488*	−0.534**	−0.221	−0.297	0.471*	0.346
Liver 20:5n-3	0.480*	0.687**	0.601**	0.644**	0.257	0.339
Liver 22:6n-3	0.544**	0.744**	0.425*	0.472*	0.112	−0.035
Liver n-3 PUFA sum	0.594**	0.566**	0.103	0.143	0.117	0.000
Liver FAS vs. ACC1	0.691**		0.958**		0.875**	

FAS: fatty acid synthase, ACC1: acetyl-CoA carboxylase-1, TAG: triacylglycerols, WAT: white adipose tissue, BM: body mass, NEFA: non-esterified fatty acids, PUFA: polyunsaturated fatty acids.

* $P < 0.05$.

** $P < 0.01$.

a continued availability of carbohydrates, shift the metabolic balance of wild bank voles towards DNL. Carbohydrate-based substrates play a central role in human DNL that is a contributor to NAFLD,⁶ suggesting a correlation between dietary carbohydrate intake and steatosis in humans.²² Thus, the mechanisms potentially responsible for the long-term regulation of DNL in voles could also have relevance in human medicine. However, the dietary hypothesis remains to be tested in the future. A multitude of factors besides food can influence the dynamics of voles, and many of these may vary pronouncedly in different phases of the cycle.²⁴ This greatly complicates the distinction of the multiple causes and effects.

The present results suggest that the fatty liver of voles is not exacerbated by DNL unlike in the previously studied American mink and NAFLD patients. In wild voles, the DNL pathway is induced at declining population density, hypothetically due to scarcity of high-quality food.

Author contributions: All authors designed the study and interpreted the results. Conducted trapping and sampling: PN, A-MM, and OH. Performed laboratory and statistical analyses: LH, PN. Wrote the manuscript: PN, A-MM. All authors approved the final version of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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