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METHODOLOGY

# Assessing regional differences in hepatocyte density in wild rodents: a key step toward reliable liver cytometry

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## Abstract

Cytometric liver traits (e.g., cell area and nuclear-cytoplasmic ratio) are sensitive indicators of hepatic function, responding to diverse endogenous and exogenous factors. However, their application in population studies requires resolving two key methodological issues: how these traits vary across liver regions and the sample size that ensures reliable analysis. We analyzed hepatocyte density (cells per tissue unit area), a countable trait inversely correlated with cell area, but less labor-intensive to measure. In wild bank voles (*Clethrionomys glareolus* (Schreber, 1780)), we quantified density of mononuclear, binuclear, and anucleate hepatocytes across the liver lobes and two functionally distinct acinar zones (afferent/periportal vs. efferent/perivenous). The total number of cells analyzed (tens of thousands) exceeded by 6–7 times the minimum sample size (≈4,000) required to detect significant differences with an effect size as small as 2%, ensuring high statistical power. Using multinomial, binomial, and Poisson regression models (according to the type of data analyzed), we assessed the effects of sex, liver mass, and region on the density and proportion of the 3 types of hepatocytes. Hepatocyte density was consistently higher in efferent zones than in afferent zones (all cell types, 4% (95% CI: 3–5%)), but did not differ significantly between lobes or sexes. Liver mass significantly influenced hepatocyte composition; the proportion of anucleate cells increased proportionally with mass (log-odds = 0.53, $p < 0.001$), suggesting that the anucleate/nuclear ratio could serve as a structural and/or functional marker. Our results suggest a link between the morphological and functional heterogeneity of hepatocytes and highlight the importance of considering the acinar zone when planning cytometric studies of the liver.

**Keywords** Liver tissue heterogeneity · Acinar zone · Cytometric analysis · Small mammals

## Introduction

The liver is a vital organ that plays a central role in metabolism and detoxification, including accumulation, transport, and elimination of toxic compounds (Treinen-Moslen 2001; Malhi et al. 2010). The morphological and functional characteristics of hepatocytes, the principal cells of the liver, serve as key indicators of the organ's physiological state and an organism's adaptive capacity to environmental stressors (Williams and Iatropoulos 2002; Pereira et al. 2006; Tête et al. 2014). These include qualitative (e.g., presence of granules, vacuoles, or deformed nuclei) and quantitative (e.g., cell size, density, and nuclear-cytoplasmic ratio) traits, which are widely used as biomarkers of toxic exposure (Pereira et al. 2006; Salińska et al. 2012, 2013; Amuno et al. 2016). Although cytomorphometry is less frequently employed in ecotoxicology than histopathology because of its labor-intensive nature, cytometric parameters often exhibit higher sensitivity to environmental pollutants, particularly heavy metals (Sánchez-Chardi et al. 2009; Davydova et al. 2023).

In murine rodents, the liver comprises six lobes: right and left medial, right and left lateral, caudate, and papillary (Fox et al. 2006). At the tissue level, the parenchyma is organized into acini – functional units centered around the terminal branches of the portal vein and hepatic artery (Rappaport et al. 1954; Gumucio 1989; Usynin and Panin 2008). Each acinus is divided into three concentric zones: afferent (or periportal, in terms of the 'classical' hepatic

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lobule), intermediate, and efferent (perivenous), reflecting the functional heterogeneity in oxygen gradients, hormone distribution, and metabolic activity. This zonation has been documented in humans and other mammals (Schmucker 1990; Jungermann and Kietzmann 1996; Rajvanshi et al. 1998; Massimi et al. 1999; Saito et al. 2013; Gebhardt and Matz-Soja 2014).

During the planning of cytomorphometric studies of the liver, a key consideration is the potential influence of the selected tissue area on the measured hepatocyte characteristics. In our previous study, we standardized the measurement procedure and aimed to minimize or, if necessary, account for the variability associated with tissue heterogeneity by restricting the analysis to precisely defined regions (Davydova et al. 2023). Although this approach ensures the reproducibility of results, it does not account for potential differences in hepatocyte morphology between different parts of the liver.

We conducted a methodological study to assess the extent of morphological heterogeneity in the hepatic tissue of rodents from natural populations and to determine how the choice of liver region affects cytomorphometric outcomes. To this end, we measured the packing density (hereafter referred to as hepatocyte density) and proportion of mono-, bi-, and anucleate hepatocytes across different lobes and acinar zones, while also evaluating the influence of sex and liver mass on these traits. Hepatocyte density is inversely correlated with cell area (the cell's projection onto the plane); however, unlike cell area, it does not require labor-intensive measurements, thus serving as a convenient parameter for detecting structural features, including pathological alterations, in liver tissues (Munguti et al. 2020). Based on the available literature (Rappaport et al. 1954; Bhatia et al. 1996; Lamers et al. 1989; Gebhardt and Matz-Soja 2014; Kietzmann 2017), we a priori expected to find an anatomical polarization of the studied parameter across the acinar zones and, conversely, assumed that conditions for it would be isotropic across the different liver lobes. In the former case, our aim was to quantify these differences in one of the most common species of wild rodents; in the latter, to verify that they were negligible (i.e., at the level of random variation) and could be disregarded in future studies.

## Materials and methods

### Animal sampling

The bank vole (*Clethrionomys glareolus* (Schreber, 1780) is a widely distributed rodent in the forest ecosystems of Eurasia (Kryštufek and Shenbrot 2022). Its high abundance, high fecundity, short life cycle, and ability to respond rapidly to various biotic and abiotic factors make it a convenient model for numerous evolutionary and ecological studies (Henttonen 2000; Crespin et al. 2002; Ledevin et al. 2010; Imholt et al. 2015; Bobretsov et al. 2017; Kshnyasev and Davydova 2021; Sørensen et al. 2023; Balčiauskas et al. 2024; Bujnoch et al. 2024; etc.). In ecophysiological and ecotoxicological research, this species has been studied across various levels of biological organization, from the cellular-tissue to the population (Damek-Poprawa and Sawicka-Kapusta 2004; Włostowski et al. 2004; Salińska et al. 2013). Therefore, the bank vole represents one of the most studied species of wild rodents.

This study used materials collected during autumn population surveys of small mammals in the undisturbed spruce–fir forests of the Middle Urals. The surveys were conducted in September 2023, a period corresponding to the peak phase of the bank vole population cycle, characterized by high spring densities of overwintered individuals and suppressed young of the year maturation. The catch index was 33 voles per 100 trap-days. By autumn, the population of the bank vole consisted mainly of immature young, which ensured maximum sample homogeneity.

Rodents were trapped using wooden live traps that were checked twice daily. Traps were lined with moss and baited, enabling animals to survive in night-time temperatures of 3–8 °C. Animals were euthanized by cervical dislocation in the laboratory, and the following measurements were recorded. The internal organs, including the liver, were weighed on a TANITA 1210-50 electronic balance (Tanita Corporation, Japan) with a precision of 0.02 mg.

Based on external (body mass and size) and internal (condition of the thymus and reproductive organs, mass of internal organs) traits, as well as the presence of tooth roots and the date of capture, the animals were classified into three reproductive-age groups: overwintered individuals, mature young of the year, and immature young of the year. This structure reflects the ontogenetic differences in boreal muroid rodents, which, in turn, ensure the redistribution of reproductive effort within the population (Novikov and Moshkin 2009). The peculiarity of these population differences lies in the fact that one part of the animals (mature young, with a maximum lifespan of 3–5 months) matures in the year of their birth, while the other (immature young, with a maximum lifespan of 13–14 months) – in the following year after overwintering (Olenev 2002, 2009).

The study sample included only immature young – 10 males and 10 females aged 2–4 months. These animals are characterized by reduced metabolism and a slower growth rate (almost twice as slow compared to mature young). This group is considered the most resilient to adverse influences, as its primary function is to preserve the population until the next breeding season. After overwintering, such individuals

rapidly mature and reach their definitive state, and their metabolic and aging rates become similar to those of mature young (Olenev 2002).

## Morphometric analysis

After fixation in 10% formalin solution, the liver was divided into 6 lobes (*lobus sinister lateralis*, *l. sinister medialis*, *l. dexter medialis*, *l. dexter lateralis*, *l. caudatus*, *l. papillaris*), with the papillary lobe excluded from the study due to its small size and fragmentation.

For microscopic examination, 3–6 paraffin sections (5–7 µm thick) were prepared from each lobe, mounted on glass slides, and stained with hematoxylin and eosin (Romeis 1953) (Fig. 1). The micromorphology of the liver (5 slides per individual) was examined using a Leica DM1000 LED microscope (Leica Microsystems, Germany) equipped with a Leica DFC 295 camera and ImageScope M software (http://www.microscop.ru). Qualitative liver changes were not assessed in this study. To avoid subjective bias in the evaluations, the analysis of the specimens was performed by a single operator in a random order, with the sample numbering concealed from them. The packing density of mono-, bi-, and anucleate hepatocytes (cell count per unit area of tissue) was determined at 630× magnification in two functionally distinct acinar zones: afferent (Aff) and efferent (Eff) zones. Areas for measurement were selected adjacent to blood vessels – the central vein (for the Eff-zone) or the vessels of the portal triad (for the Aff-zone) (Fig. 2). Five fields of view were examined for each liver lobe in both acinar zones. Only one field of view was selected per area. In total, 50 fields of view were analyzed per individual, each covering an area of 10,000 µm². A total of 30,628 cells were counted, including 20,883 (68%) mononuclear hepatocytes, 3,937 (13%) binuclear hepatocytes, and 5,808 (19%) anucleate hepatocytes.

## Statistical analysis

To prevent selection bias when choosing fields of view for analysis, hepatocyte counts were summed across five randomly selected fields. Variability in the proportions of the three hepatocyte types as a function of separate factors (while controlling others) was examined using multinomial logistic regression. We also considered the option of converting the original polytomous (three-level) dependent variable into a binary variable. This involved either merging the three cell types into two categories, or excluding one type, followed by employing an easily interpretable binomial logistic regression.

The predictors included sex (male = 1), liver lobe and acinar zone (#1 and #3), and liver mass (mg). Liver lobes were parameterized as a categorical variable with five levels, and sex and acinar zone were coded using indicator (0, 1) variables. To allow for meaningful comparison of effect sizes (regression coefficients) across categorical and continuous predictors, liver mass was transformed as $(X_i - X_{min})/(X_{max} - X_{min})$, where $X_{max}$ = 1968 mg and $X_{min}$ = 1092 mg. After exponential transformation, log-odds ratios (LOR, i.e., regression coefficients) with absolute values < 0.1 can be directly interpreted as log-risk ratios (Agresti 2007). For count data (i.e., number of cells), Poisson regression was applied, with cell type parameterized as an additional predictor.

The inclusion of predictors in the statistically optimal model was evaluated using the Consistent Akaike

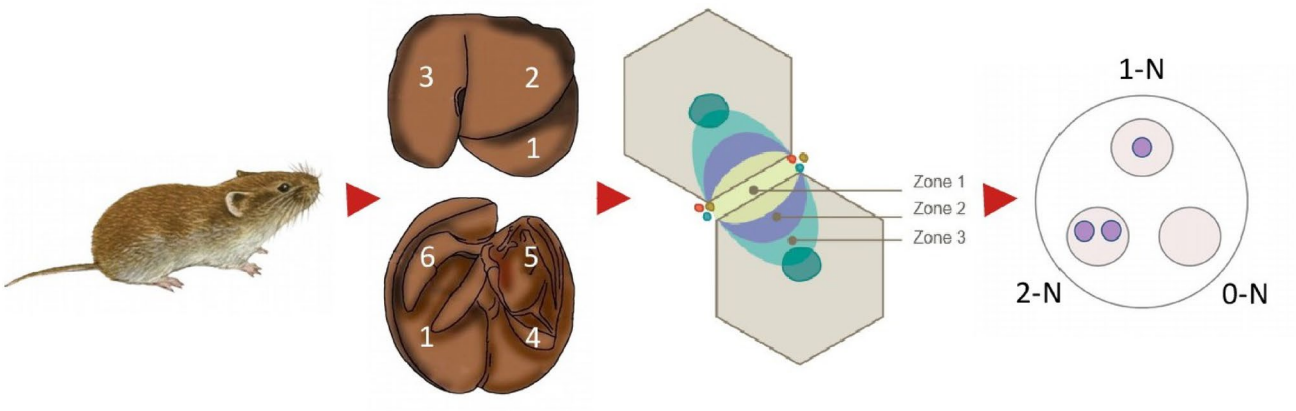


**Fig. 1** Study design for hepatocyte density analysis in the bank vole liver. Macroscopic view of the liver from the diaphragmatic (top) and visceral (bottom) surfaces. Liver lobes: 1 – left lateral, 2 – left medial, 3 – right medial, 4 – right lateral, 5 – caudate, 6 – papillary (not sampled). Zones of acinus: afferent (Zone 1, adjacent to the portal triad) and efferent (Zone 3, adjacent to the central vein). Hepatocyte types: mononuclear (1-N), binuclear (2-N), anucleate (0-N). For morphometric analysis, a separate tissue block was prepared from each of the five lobes; from each block, 3–6 sections were mounted per slide, resulting in five analyzed slides per animal. Bank vole image and acinus schematic adapted from open Internet sources

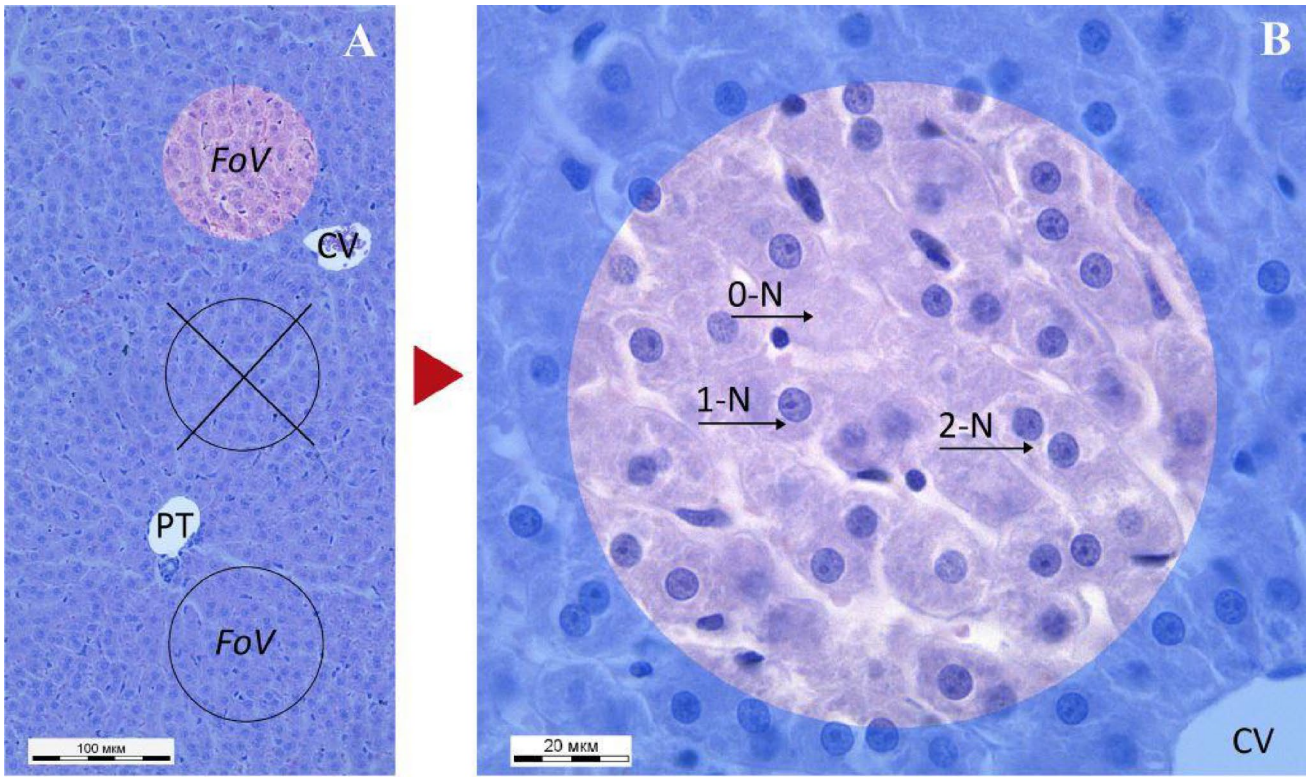


**Fig. 2** Scheme for selecting liver tissue areas for cytometry (5 lobes × 2 acinar zones × 5 fields of view): **A** Fields of view (*FoV*) were selected based on proximity to the portal triad (PT) for the afferent zone or the central vein (CV) for the efferent zone. One *FoV* was analyzed per zone. This procedure was repeated five times for each zone. The distance between the PT and CV vessels was sufficient to ensure that the fields of view adjacent to them did not overlap. **B** Within each field of view, the number of mononuclear (1-N), binuclear (2-N), and anucleate (0-N) hepatocytes was counted. The immediate perivascular cell layer (one-cell-thick) was not included in the field of view

**Table 1** Selection of optimal (min CAIC) multinomial logit regression model for hepatocyte density

| Model rank | Predictors | K | –2LL | LR(K–1) | CAIC | Δ | w |
|---|---|---|---|---|---|---|---|
| 1 | Liver mass, Acinar zone | 3 | 29649.17 | 102.04 | 29683.16 | 0 | 0.545 |
| 2 | Liver mass | 2 | 29660.86 | 90.36 | 29683.52 | 0.36 | 0.455 |
| 3 | Acinar zone | 2 | 29739.69 | 11.53 | 29762.35 | 79.19 | ≈0.000 |
| 4 | $H_0$ | 1 | 29751.22 | | 29762.55 | 79.39 | ≈0.000 |

K – number of predictors, including $b_0$ (intercept); –2LL – negative twice maximum of the log-likelihood of the current model, measuring model goodness-of-fit (lower values indicate better fit); LR(df = K–1) – Likelihood Ratio statistic for comparing models by testing the null hypothesis ($H_0$) that the simpler model fits as well as the complex one, follows a $X^2$ distribution; Δ difference in CAIC between the current and best model (lowest CAIC value); w – model weight, representing the relative likelihood of the model given the data (higher values indicate the better models)

Information Criterion (CAIC): $CAIC = -2LL + K[1 + \ln(N)]$; the model with the lowest CAIC value was selected as optimal. To estimate the minimum sample size required to detect significant effects, we conducted a post-hoc power analysis. The data were modeled using the software package Statistica 8.0 (StatSoft, Inc. 2007).

## Results

Sex and liver lobe showed no significant effect on hepatocyte density (multinomial model: Type-III LR, $X^2_{sex}(2) = 3.57$, $p = 0.17$ and $X^2_{lobe}(8) = 8.91$, $p = 0.35$; binomial model: $X^2_{sex}(1) = 0.68$, $X^2_{lobe}(4) = 1.43$). Therefore, these predictors were excluded from subsequent statistical modelling. Model comparison identified liver mass and the acinar zone as the only statistically significant predictors (Table 1).

The proportion of hepatocyte types varied with liver mass, whereas the acinar zone influenced the ratio of

**Table 2** Effects of liver mass and acinar zone on hepatocyte group proportions: multinomial and binomial logit regression results

| Predictors | *b* | SE | Wald | $p \leq$ | Exp(*b*) | –95% CI | +95% CI |
|---|---|---|---|---|---|---|---|
| Multinomial logit regression | | | | | | | |
| Mononuclear hepatocytes | | | | | | | |
| $b_0$ | **1.47** | **0.025** | **3350.91** | **0.001** | **4.36** | **4.15** | **4.58** |
| Liver mass (0–1) | **–0.52** | **0.054** | **93.61** | **0.001** | $\mathbf{1.68^{-1}}$ | $\mathbf{1.87^{-1}}$ | $\mathbf{1.52^{-1}}$ |
| Acinar zone (Aff) | **–0.06** | **0.015** | **15.08** | **0.001** | $\mathbf{1.06^{-1}}$ | $\mathbf{1.09^{-1}}$ | $\mathbf{1.03^{-1}}$ |
| Binuclear hepatocytes | | | | | | | |
| $b_0$ | **–0.23** | **0.035** | **41.68** | **0.001** | $\mathbf{1.25^{-1}}$ | $\mathbf{1.34^{-1}}$ | $\mathbf{1.17^{-1}}$ |
| Liver mass (0–1) | **–0.43** | **0.076** | **32.39** | **0.001** | $\mathbf{1.54^{-1}}$ | $\mathbf{1.79^{-1}}$ | $\mathbf{1.33^{-1}}$ |
| Acinar zone (Aff) | –0.01 | 0.021 | 0.18 | 0.7 | $1.01^{-1}$ | $1.05^{-1}$ | 1.03 |
| Binomial logit regression | | | | | | | |
| Mononuclear or binuclear hepatocytes | | | | | | | |
| $b_0$ | **1.64** | **0.025** | **4300.67** | **0.001** | **5.16** | **4.91** | **5.42** |
| Liver mass (0–1) | **–0.51** | **0.053** | **92.11** | **0.001** | $\mathbf{1.66^{-1}}$ | $\mathbf{1.84^{-1}}$ | $\mathbf{1.50^{-1}}$ |
| Acinar zone (Aff) | **–0.05** | **0.015** | **11.69** | **0.001** | $\mathbf{1.05^{-1}}$ | $\mathbf{1.08^{-1}}$ | $\mathbf{1.02^{-1}}$ |

Statistically significant effects ($p<0.01$) are highlighted in bold

$b_0$ – reference group (anucleate cells; Eff-zone of the acinus; liver mass transformed to range: 0–1); *b* – regression coefficient; SE – standard error; Wald – $X^2(1)$ test statistic; *p* – significance level; Exp(b) – odds ratio; CI – confidence interval

**Table 3** Hepatocyte group proportions: dichotomized logit regression results (third cell type excluded)

| Predictors | Hepatocytes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Anucleate/Binuclear | | | Anucleate/Mononuclear | | | Binuclear/Mononuclear | | |
| | *b* | SE | Wald | *b* | SE | Wald | *b* | SE | Wald |
| $b_0$ | **0.25** | **0.036** | **48.93** | **–1.48** | **0.026** | **3169.49** | **–1.71** | **0.030** | **3209.18** |
| Sex (male) | –0.058 | 0.043 | 1.81 | –0.004 | 0.016 | 0.07 | –0.03 | 0.018 | 3.57 |
| Liver mass (0–1) | **0.37** | **0.077** | **23.53** | **0.53** | **0.056** | **87.95** | 0.12 | 0.068 | 3.15 |
| Acinar zone (Aff) | 0.01 | 0.021 | 0.26 | **0.06** | **0.015** | **15.29** | **0.05** | **0.017** | **7.67** |

Statistically significant effects ($p<0.01$) are highlighted in bold

$b_0$ – reference group (anucleate cells; Eff-zone of the acinus; liver mass transformed to range: 0–1); *b* – regression coefficient; SE – standard error; Wald – $X^2(1)$ test statistic; *p* – significance level; Exp(b) – odds ratio; CI – confidence interval

anucleate cells to both mononuclear and total nucleated cells (Table 2). Despite the high statistical significance, effect sizes were modest: the odds of encountering mononuclear hepatocytes in the Aff-zone of the acinus were 1.06 (95% CI: 1.03–1.09) times lower than in the Eff-zone. Compared to anucleate cells, the encounter frequency of mononuclear hepatocytes was 4.36 (4.15–4.58) times higher, while that of binuclear cells was 1.25 (1.34–1.17) times lower.

Liver mass consistently and negatively affected the odds of detecting nucleated hepatocytes, and an increase of approximately 1 g was associated with a 1.5–1.7-fold decrease in the odds of encountering mono- or binuclear cells (Table 2).

Analysis of simplified censored data (excluding one hepatocyte group) revealed additional contrasts in hepatocyte-type proportions (Table 3). Binuclear cells showed the least variability, with only 5% dependence on the acinar zone. In contrast, anucleate cell proportions were significantly influenced by both the acinar zone and liver mass; they were higher in the Aff-zone than in the Eff-zone and increased progressively with liver mass.

Analysis of the absolute number of cells (*n* summed across the five fields of view) confirmed the patterns described above. In the Aff-zone, the total number of all hepatocyte types was 4% (3–5%) lower than in the Eff-zone (Table 4). An increase in liver mass by approximately 1 g was associated with a 1.2-fold reduction in the total hepatocyte count (Fig. 3). As expected, mononuclear hepatocytes were the most abundant, occurring 3.6 (3.5–3.7) times more frequently than the anucleate cells. Binuclear cells were the least frequent, being 1.5 (1.4–1.5) times less common than anucleate cells.

Power analysis showed that to detect statistically significant differences comparable in magnitude to the contrast between acinar zones (with an effect size of approximately 2%, as observed for the proportion of anucleate cells), a sample size of ≈4 000 cells is sufficient. The number of cells that were counted significantly (6–7 times) exceeded the minimum required size.

**Table 4** Effects of liver mass and acinar zone on absolute hepatocyte counts: Poisson regression with log-link, c = 1

| Predictors | *b* | SE | Wald | *p* ≤ | Exp(*b*) | –95% CI | +95% CI |
|---|---|---|---|---|---|---|---|
| $b_0$ | 3.43 | 0.015 | 50647.57 | 0.001 | 30.87 | 29.96 | 31.80 |
| Mononuclear cells | 1.28 | 0.015 | 7441.69 | 0.001 | 3.60 | 3.49 | 3.70 |
| Binuclear cells | –0.39 | 0.021 | 354.73 | 0.001 | $1.48^{-1}$ | $1.54^{-1}$ | $1.42^{-1}$ |
| Liver mass (0–1) | –0.17 | 0.021 | 61.43 | 0.001 | $1.18^{-1}$ | $1.23^{-1}$ | $1.13^{-1}$ |
| Acinar zone (Aff) | –0.04 | 0.006 | 46.68 | 0.001 | $1.04^{-1}$ | $1.05^{-1}$ | $1.03^{-1}$ |

Statistically significant effects ($p < 0.01$) are highlighted in bold

$b_0$ – reference group (anucleate cells; Eff-zone of the acinus; liver mass transformed to range: 0–1); *b* – regression coefficient; SE – standard error; Wald – $X^2(1)$ test statistic; *p* – significance level; Exp(b) – odds ratio; CI – confidence interval

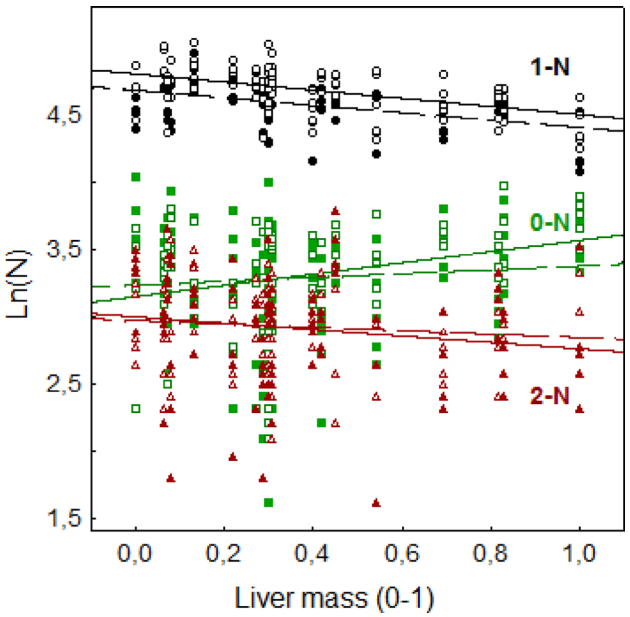


**Fig. 3** Relationship between liver mass (normalized scale 0–1) and the natural logarithm of the total hepatocyte count (Ln(N)). Each data point represents the sum of counts across five fields of view. Symbols denote hepatocyte types: mononuclear (1-N, circles), binuclear (2-N, triangles), anucleate (0-N, squares). Fill denotes acinar zones: afferent (Aff, open symbols) and efferent (Eff, filled symbols). Each vertical cluster of six symbols represents one animal ($n = 20$)

## Discussion

### Macromorphology of the bank vole liver

Multilobarity is a characteristic feature of mammalian liver structure; however, the details of its architecture and the degree of lobulation vary significantly among species (Romer and Parsons 1986). The macromorphology of the human liver and that of economically important animals is well-documented, with different authors distinguishing from 2 to 8 lobes. The bank vole is one of those wild species for which a detailed description of liver macromorphology is available (European bank vole, 1981).

As in other muroid rodents, the liver of the bank vole is located in the abdominal cavity almost symmetrically relative to the body's midline. It lacks the median and quadratic lobes characteristic of humans and large animals, and the left and right medial lobes essentially represent a single lobe divided by an incisure (Fox et al. 2006; Kruepunga et al. 2019). This structural reduction is associated with the small body size of the animal and its fusiform (elongated and rounded) body shape, which is determined by a burrowing lifestyle. The small, dome-shaped volume of the abdominal cavity (the dome apex is formed by the diaphragm) determines both the almost symmetrical position of the liver relative to the body axis and the poor differentiation of its medial lobes. It should be noted that the liver structure of different rodent species differs only in minor details, primarily pertaining to the visceral surface of the organ (e.g., the shape and size of the papillary lobe).

### Concepts of the classical hepatic lobule and the acinus

According to Sasse et al. (1992), the difficulty in establishing a correspondence between the structural and functional units of the liver stems from the duality of its functions: on the one hand, the liver is an exocrine gland that secretes bile; on the other, its microcirculatory pattern resembles that of endocrine organs, as it involves the release of various substances into the blood. The microscopic architecture of the liver is explained by two main concepts of its structural-functional unit – the classical hepatic lobule and the hepatic acinus, which are recognized by researchers to varying degrees (Lamers et al. 1989; Bhatia et al. 1996; Saxena 1999; Kruepunga et al. 2019). The classical hepatic lobule (*lobuli hepatis*) is defined by structural features, while the acinus is defined based on microcirculatory blood flow. In contrast to the hepatic lobule, a concept that has existed for over a century and a half, the acinus was described relatively recently (Rappaport et al. 1954). The classical lobule highlights the portocentral metabolic gradient and illustrates the processes of blood flow and bile excretion (Saxena et al. 1999). The acinus concept emphasizes the conditions of microcirculation, which determine differences in the blood supply to different zones and, consequently, in their oxygen and nutrient levels (Rappaport and Wilson 1958; Rappaport

1973; Lamers et al. 1989). While acknowledging the value of both models, we leveraged the zonal differentiation of the acinus to contrast its functional principle with the morphological techniques and metrics employed in our work.

It should be noted that quantitative liver parameters at the organ (total organ volume), tissue (volumetric density of parenchyma, stroma, and sinusoids), and cellular (total number, density, mean volume of hepatocytes and their nuclei, proportion of binuclear cells) levels have been previously assessed in rodents (laboratory rats and mice) (Jack et al. 1990; Altunkaynak and Ozbek 2009; Karbalay-Doust and Noorafshan 2009). However, those studies did not aim to evaluate regional differences in these parameters.

## Functional and morphological heterogeneity of hepatic tissue

According to the concept of functional (metabolic) zonation of the liver (Katz and Jungermann 1976), the afferent (Aff) zone of the acinus in healthy tissue is primarily the site of numerous catabolic and synthetic processes. These include oxidative phosphorylation, β-oxidation of fatty acids, gluconeogenesis, and deamination of amino acids (e.g., catabolism of histidine and serine). This zone is also responsible for bile formation and secretion, the synthesis of major blood plasma proteins (including albumin and fibrinogen), urea formation, as well as cholesterol synthesis and degradation. In contrast, the efferent (Eff) zone is dominated by glycolytic and biosynthetic pathways. Key processes here comprise glycolysis, de novo lipogenesis, and the synthesis of glutamine (associated with ammonia detoxification), glycogen from glucose, and bile acids. This zone is also the primary site for ketogenesis and biotransformation reactions (metabolism of xenobiotics) (Colnot and Perret 2011; Schleicher et al. 2015; Matz-Soja 2019). The fact that these numerous and often opposing metabolic processes occur simultaneously in closely adjacent zones points to a complex regulatory network governing liver zonation, which is underpinned by specific genetic mechanisms (Katz and Jungermann 1976; Bhatia et al. 1996).

Differences in metabolic activity between the zones of the hepatic acinus explain the heightened sensitivity of the Eff-zone to various factors (Rappaport et al. 1954; Gumucio 1989; Usynin and Panin 2008). Differences in the density (and size) of hepatocytes may also reflect their adaptation to distinct metabolic functions in different zones of the acinus. In other words, the morphological features of hepatocytes may be correlated with their functional heterogeneity.

Furthermore, the varying hepatocyte density in the Aff- and Eff-zones could be attributed to the structural and ultrastructural features of the hepatic tissue. For example, the lower hepatocyte density in the Aff-zone may be associated with zonal differences in the sinusoids and bile capillaries, as the volume of the liver occupied by these structures in the Aff-zone is greater than that in the Eff-zone (Schmucker et al. 1978; Qin and Crawford 2018).

In contrast to functional heterogeneity, evidence for morphological heterogeneity in liver tissue is significantly more limited. Zonal differences have been identified in ultrastructural studies (number of mitochondria and peroxisomes, area of smooth endoplasmic reticulum, etc.) (Loud 1968; Ma and Biempica 1971; Asada-Kubota et al. 1982), in the distribution of cells with different ploidy and number of nuclei (Sasse et al. 1992; Rajvanshi et al. 1998), and in hepatocyte size (Bhatia et al. 1996; Fomenko et al. 2019).

Interlobar heterogeneity of the liver has been demonstrated in the accumulation of heavy metals: in newborns, Fe and Cu accumulated predominantly in the left lobe (Faa et al. 1987, 1994), whereas in an adult patient with Wilson's disease at the cirrhosis stage, excess Cu was deposited in the right lobe (Faa et al. 1995). Studies in rats have shown that liver lobes differ in their susceptibility to hepatocellular carcinoma: after diethylnitrosamine administration, the incidence was highest in the left lobe and the right median lobe compared to the right anterior lobe (Richardson et al. 1986).

Overall, many authors acknowledge that the factors determining liver tissue heterogeneity are not fully understood and continue to work on identifying the inducers and regulators of this phenomenon (Gebhardt and Matz-Soja 2014; Sato et al. 2014; Birchmeier 2016).

### Liver mass and hepatocyte density

Experimental studies indicate that liver mass changes due to the accumulation or depletion of carbohydrates and fats in hepatocytes, which, in turn, can depend on the animals' environmental conditions (Bezborodkina et al. 2022; Xu et al. 2025). For example, it has been shown that changes in hepatocyte size are responsible for seasonal variations in liver mass in rodents (Bonda-Ostaszewska and Włostowski 2015). In our study, we evaluated for the first time the influence of liver mass in a rodent from a natural population on the ratio of different hepatocyte types and their total number. The simultaneous decrease in the frequency of both mononuclear and binuclear hepatocytes, coupled with higher detection rates of anucleate (degenerative) cells in animals with increased liver mass (Table 2), could reflect either seasonal metabolic adaptation preceding winter or pathological alterations. However, distinguishing between these possibilities requires additional qualitative analyses of liver tissue morphology and biochemistry, which were not performed in this study. Future investigations should include examination

of livers from wintering and post-winter animals for a comparative assessment of these seasonal adaptations.

The decrease in the total number of hepatocytes, alongside an increase in absolute liver mass, is likely due to cellular hypertrophy driven by the accumulation of cytoplasmic components (glycogen and/or lipids) in the pre-winter period. These changes may reflect a functional adaptation of the organ, including the activation of enzyme systems and enhanced detoxification activity, which are necessary for the seasonal transition of voles to a winter diet rich in secondary plant metabolites (phenols and terpenes). Surprisingly, liver mass proved to be a stronger factor influencing hepatocyte density than zonal histological gradient. This promising finding brings us back to the simple yet effective method of morphophysiological indicators, proposed by Schwartz et al. (1968), which is widely used in ecological studies. This method allows for assessing the physiological state of individuals in a population based on the mass and/or relative size of internal organs, particularly the liver. It should be noted that for the bank vole, the mean value of the liver index (the ratio of organ mass to body mass, expressed in ‰) is known to range from 60 to 73‰ (for adult males). The variability of this index across different populations and under different environmental conditions has also been described (European bank vole 1981; Ivanter et al. 1985; Nieminen et al. 2015).

## Regional differences in mononuclear, binuclear and anucleate hepatocytes

It is believed that binuclear hepatocytes, formed as a result of incomplete mitosis of mononuclear cells, reflect the intensity of regenerative processes in the hepatic tissue (Brodsky et al. 2024). However, the weak effects of the acinar zone and liver mass on their density limit the diagnostic value of this parameter for assessing hepatic tissue alterations. In contrast, a shift in the proportion of anucleate cells, which strongly depends on both the acinar zone and liver mass (Table 3), may serve as an informative marker and potential indicator of tissue changes.

Mononuclear hepatocytes were the most numerous among all cell types. The total number of all cell types was similar across different liver lobes but differed between the Aff- and Eff-zones of the acinus. Our observations are consistent with data on hepatocyte density in piglets (Junatas et al. 2017). In a stereological study of piglet liver, these authors found no significant differences in hepatocyte size, nuclearity, or density among the six liver lobes but revealed significant intralobular differences (relative to the distance of parenchymal areas to the liver's large vessels) (Junatas et al. 2017). We also recorded significant differences at the acinar level, whereas no differences were found between the lobes. These results suggest that, in morphometric analyses, it is crucial to account for the zonal localization of hepatocytes, whereas the choice of the liver lobe is of lesser importance. This approach simplifies the sample selection and broadens the experimental design possibilities.

## Study limitations and future perspectives

Fundamental limitation of the morphometric approach used in this study is its reliance on two-dimensional projections of hepatocytes, which may not fully capture their three-dimensional morphology. While this is a common constraint in routine histological analysis, it is important to note that specialized stereological techniques exist specifically to extrapolate three-dimensional parameters from two-dimensional sections (Jack et al. 1990; Junatas et al. 2017).

Previous studies on laboratory animals (rats and mice) have revealed sex-dependent functional differences at the acinar level associated with variations in protein and enzyme concentrations (Sirma et al. 1996; Massimi et al. 1999). In our study, hepatocyte density was comparable between males and females, likely because of the immaturity of the animals. We hypothesized that sex-related morphological differences in hepatocytes might become detectable with sexual maturation (i.e., increased gonadal steroid levels).

Despite the relative age homogeneity of our sample, the statistical power of the analysis was sufficient to detect minor (<4%) differences in the hepatocyte density. This suggests that by expanding the experimental design, for example, by addition contrasting levels of other factors (e.g., reproductive and age status, environmental pollution), even weaker effects could be detected. Moreover, because the number of cells quantified far exceeds the threshold required for statistical significance, future large-scale cytomorphometric studies (e.g., population-level analyses) could be optimized by reducing the number of cells examined. Therefore, it is more productive to focus efforts on increasing the number of subjects rather than the number of cells per subject, since the variance between animals is the biologically relevant measure for population studies. Measuring excessive numbers of cells from a few individuals only improves the precision of estimates for those specific animals but does not allow for reliable generalization to the population level.

Although our study focused on the bank vole as a convenient model for ecological research, the conclusions drawn may have broader methodological relevance for liver studies in other mammalian species. The approach to standardizing sampling protocols and the insights into the relationship between liver mass and hepatocyte composition could be applied to the study of liver cytometric traits in a wide range of wild and laboratory species.

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**Data availability** Raw data not presented in this article can be made available upon reasonable request to the authors.

## Declarations

**Conflict of interest** The authors declare no competing interests.

**Ethical approval** All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the Institute of Plant and Animal Ecology RAS (Protocol No 3 of 18/12/2014).

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