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Applying the Anna Karenina principle for wild animal gut microbiota: temporal stability of the bank vole gut microbiota in a disturbed environment

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Abstract

1. Gut microbiota play an important role in host health. Yet, the drivers and patterns of microbiota imbalance (dysbiosis) in wild animals remain largely unexplored.
2. One hypothesised outcome of stress on animal microbiomes is a destabilised microbial community that is characterised by an increase in inter-individual differences compared with microbiomes of healthy animals, which are expected to be (i) temporally stable and (ii) relatively similar among individuals. This set of predictions for response of microbiomes to stressors is known as the *Anna Karenina principle* (AKP) for animal microbiomes.
3. We examine the AKP in a wild mammal inhabiting disturbed environments by conducting a capture-mark-recapture survey of bank voles (*Myodes glareolus*) in areas that contrast in levels of radionuclide contamination (Chernobyl, Ukraine).
4. Counter to key predictions of the AKP, bank voles that are not exposed to radionuclides harbour variable (increased inter-individual differences) and temporally dynamic gut microbiota communities, presumably tracking the natural spatio-temporal variation in resources. Conversely, bank voles exposed to radionuclides host more similar gut microbiota communities that are temporally stable, potentially due to a dysbiosis or selection (on host or bacteria) imposed by chronic radiation exposure.

5. The implication of these data is that environmental stress (radiation exposure) can constrain the natural spatial and temporal variation of wild animal gut microbiota.

Keywords: Anna Karenina principle, anthropogenic disturbance, Chernobyl, environmental stress, gut dysbiosis, radiation exposure, stable isotope analysis

Introduction

Animal gastrointestinal tracts harbour complex communities of microbes, known as microbiota, that can provide diverse benefits to their host, such as defending against pathogens (Pickard, Zeng, Caruso, & Núñez, 2017), regulating the immune system (Hooper, Littman, & Macpherson, 2012), and supplying essential metabolites from otherwise indigestible foodstuffs (Gentile & Weir, 2018). As delivery of these services impacts host health, and often adaptive potential (Alberdi, Aizpurua, Bohmann, Zepeda-Mendoza, & Gilbert, 2016), there is a clear advantage for a host to exert some control over the composition of its microbiota (Rosenberg & Zilber-Rosenberg, 2018). Yet, vertebrate microbiota are characterised by considerable inter-individual variability, due to factors such as diet (Maurice et al., 2015; Ren et al., 2017), environment (Amato et al., 2013), and host genotype (Bolnick et al., 2014). Quantifying the composition and drivers of a healthy and dysbiotic (imbalanced) microbiota is essential to understand the significance of host-microbiota interactions.

The composition of animal microbiota can be affected by diverse stressors, for example exposure to predators (Zha, Eiler, Johansson, & Svanbäck, 2018), parasites (Leung, Graham, & Knowles, 2018), and social disruption (Karl et al., 2018). Moreover, anthropogenic stressors can alter host-associated microbial communities via habitat modification,

degradation, and contamination (reviewed by Rocca et al., 2019; Trevelline, Fontaine, Hartup, & Kohl, 2019). Under some circumstances, stress can alter gut microbial communities through displacement of sensitive taxa, producing deterministic changes (location effects) in microbiota composition compared with control (unstressed) individuals (Gerassy-Vainberg et al., 2018; Marin et al., 2017; Richardson et al., 2018). Alternatively, stress and diseases can increase variance of microbiota (dispersion effects), resulting in higher inter-individual differences due to microbiome instability (Earley et al., 2015; Halfvarson et al., 2017; Moeller et al., 2013; Wu et al., 2016). A framework to describe such impacts of host stress on microbiota structure proposes that healthy animal microbiomes are characterised by (1) relatively high similarity among individuals and (2) temporal stability. Exposure to a stressor can disrupt normal mechanisms of microbiome control and thus releases constraints on the microbiota, whose composition can change in a stochastic fashion: under such circumstances, the microbial communities of stressed animals are characterised by high inter-individual microbiome variability and temporal changes compared with healthy individuals (Zaneveld, McMinds, & Vega Thurber, 2017). Zaneveld et al. (2017) called this the Anna Karenina principle (AKP) following the opening line of Tolstoy's *Anna Karenina*: '*All happy families are alike; each unhappy family is unhappy in its own way*'. While the AKP is important for diagnostics of microbiome dysbiosis under exposure to stress in biomedical research (in humans and laboratory animals), this idea remains largely unexplored in wild animals, which could provide a more broad ecological relevance (Zaneveld et al., 2017).

Human activities, such as processes associated with uranium mining, nuclear waste treatment, and nuclear tests, have left many areas worldwide that are contaminated by radionuclides (Lourenço, Mendo, & Pereira, 2016). Animals inhabiting the Chernobyl

Exclusion Zone (CEZ), an approximately 4,700 km^2 area affected by radioactive fallout (principally ^{90}Sr and ^{137}Cs radionuclides) from the Chernobyl accident in Ukraine in 1986, provide unique opportunities to study biological impacts of exposure to environmental radionuclides (Møller & Mousseau, 2006). Harmful effects of chronic exposure to radiation have been documented across multiple biological levels of organisation (Lourenço et al., 2016), and wildlife within the CEZ is no exception. For example, radiation exposure within the CEZ has a negative effect on the abundance and diversity of birds (Morelli, Benedetti, Mousseau, & Møller, 2018), yet little notable effects on the abundance of large mammals (Deryabina et al., 2015). At the organismal level, wildlife affected by the Chernobyl fallout exhibit a suite of phenotypic maladies such as signs of aspermy, reduced sperm motility (Møller, Bonisoli-Alquati, Mousseau, & Rudolfsen, 2014), and smaller brain size (measured as head volume) (Møller, Bonisoli-Alquati, Rudolfsen, & Mousseau, 2011). At the molecular level, exposure to radionuclides is associated with elevated DNA damage (Bonisoli-Alquati et al., 2010), increased oxidative stress (Einor, Bonisoli-Alquati, Costantini, Mousseau, & Møller, 2016), and/or chromosomal aberrations (Lourenço et al., 2016).

Notably, little is known about the effects of radiation on microorganisms and few available studies returned conflicting results. For example, some studies suggest that radionuclide contamination has negative or no effect on the diversity of free-living microbes in Chernobyl (Ragon, Restoux, Moreira, Møller, & López-García, 2011; Romanovskaya, Sokolov, Rokitko, & Chernaya, 1998), whereas others conclude that microbes within the CEZ show signs of radioresistance (Hoyos-Hernandez et al., 2019; Theodorakopoulos et al., 2017), yet with no inference on the wider consequences of radiation exposure on environmental microbial reservoirs. Evidence that radiation has little influence on the pool of microbes in the external environment within the CEZ is, however, derived from the skin microbiota of

rodents, which despite being primarily sourced from the environment (Ross, Rodrigues Hoffmann, & Neufeld, 2019), show little difference in diversity and composition between contaminated and uncontaminated areas within the CEZ (Lavrinienko, Tukalenko, Mappes, & Watts, 2018). With few exceptions, the effects of radiation on the microbial communities associated with wildlife inhabiting the CEZ remain largely unexplored.

The bank vole *Myodes glareolus* (Schreber, 1780) is a small rodent that is an important mammalian model of the biological effects of exposure to radiation, because (1) it is common within and outside the CEZ, (2) it shows limited (~1 km) dispersal (Kozakiewicz, Chołuj, & Kozakiewicz, 2007), and (3) it burrows in soil and eats contaminated foods including herbs, seeds, roots, fungi, and invertebrates (Calandra et al., 2015). Hence, bank voles inhabiting contaminated areas in the CEZ are chronically exposed to substantial (~1.3 mGy/d) doses of radiation (Beresford et al., 2018). In contaminated areas within the CEZ, bank vole population densities are lower than in uncontaminated areas and female bank voles have reduced litter sizes (Mappes et al., 2019). Also, voles caught from contaminated areas have an increased frequency of cataracts (Lehmann, Boratyński, Mappes, Mousseau, & Møller, 2016). Bank voles exposed to elevated levels of radionuclides (>10 µGy/h) within the CEZ upregulate some genes associated with DNA damage response (Jernfors et al., 2018). Furthermore, in these areas, animals demonstrate an apparent failure to control several homeostasis parameters, evident as (1) a reduced cohesiveness of gene co-expression networks (Kesäniemi, Jernfors, et al., 2019), (2) weak or degraded intra-individual correlation in telomere length among tissues (Kesäniemi, Lavrinienko, et al., 2019), and (3) altered mitochondrial homeostasis (breakdown of the association between the mitochondrial DNA copy number and genes that regulate mitochondrial biogenesis) (Kesäniemi et al., 2020). Theory predicts that environmental challenge becomes a stressor if it results in failure

of the organism to control a critical variable (Del Giudice et al., 2018). Given the multiple lines of evidence listed above, we hypothesised that bank voles inhabiting areas contaminated by environmental radionuclides within the CEZ experience elevated levels of stress (here defined as a condition of failure to control a critical variable) compared with animals from uncontaminated areas. This provides an interesting test of the AKP and a more general examination of the effect of anthropogenic habitat alteration on wildlife microbiota.

Inhabiting areas contaminated by radionuclides is associated with a marked change in the gut microbiota composition in bank voles, notable as a reduction in the proportion of the *S24-7* family (Bacteroidetes) and an increase in several members of the Clostridiales order (Firmicutes), including *Ruminococcaceae* and *Lachnospiraceae* (Lavrinienko, Mappes, et al., 2018; Lavrinienko, Tukalenko, et al., 2018). While these cross-sectional studies indicate that bank voles inhabiting areas contaminated by radionuclides can be identified by their distinct gut microbiota, an appropriate test of the AKP requires temporal sampling to quantify how stress affects microbiota stability within individuals over time (Zaneveld et al., 2017).

Our aim here was to test for signs of the AKP in a wild mammal by quantifying spatial and temporal variation in the gut microbiota of bank voles experiencing different levels of environmental stress. We thus analysed the faecal microbiota of wild bank voles from a capture-mark-recapture (CMR) study that was conducted in areas of the CEZ that differed in the level of radionuclide contamination, which we hypothesised induces stress (Figure 1; hereafter treatment). If the AKP is applicable to our study system, then the gut microbiota of bank voles experiencing stress (radiation exposure) would be characterized by an increase in inter-individual differences and a lack of temporal stability.

Materials and Methods

Study design and sampling

Bank voles (n=84) were caught at 27 sites within the CEZ (Figure 1a, Table S1) during May-June, 2016, by live-trapping (Lavrinienko, Mappes, et al., 2018). Ambient radiation dose rate was measured near each trap location at 1 cm above the ground using a hand-held Geiger counter (see Supporting Information for methods). Contaminated areas (Chernobyl High radiation, hereafter referred to as CH, n=18 sites; mean radiation level=28.6 μGy/h; range, 10-123.2 μGy/h) had significantly elevated levels of radiation compared with uncontaminated areas (Chernobyl Low radiation, hereafter referred to as CL, n=9 sites; mean=0.25 μGy/h; range 0.17-0.32 μGy/h; χ^2=43.57, p<0.05, Kruskal–Wallis test), where the level of radiation did not significantly differ from elsewhere in Ukraine (Lavrinienko, Tukalenko, et al., 2018). The contaminated and uncontaminated trapping sites replicates (CH1-2, CL1-2) were located in geographically distinct areas, as they were separated by distances of 20-30 km that exceed the typical seasonal dispersal capability of bank voles of approximately 1 km (Figure 1a; Kozakiewicz et al., 2007).

After capture, bank voles were transferred to the field laboratory within the CEZ, and placed in individual ethanol-sterilised cages. Animals were monitored for up to 2 hours and immediately after defecation, faecal pellets were frozen at -20°C, and stored at -80°C prior to DNA extraction (Lavrinienko, Mappes, et al., 2018). Subsequently, morphometric measures were recorded and animals were marked using subcutaneous transponder tags for identification upon recapture (see Supporting Information for methods). As the CEZ presents a mosaic of contaminated and uncontaminated areas sometimes separated by distances of ~200 m (Beresford, Scott, & Copplestone, 2019), we established whether trapping site can represent an animal's radiation exposure by implanting each bank vole with a lithium fluoride

thermoluminescent dosimeter (TLD, CHP Dosimetry) that measures absorbed external radiation dose. In addition, upon capture and recapture every individual was subject to γ-spectrometry (SAM 940, Berkeley Nucleonics) to estimate whole-body radionuclide (^{137}Cs) burden and internal radiation exposure (see Supporting Information for methods). As an estimate of condition (a proxy for stress), we calculated a body condition index (BCI) as the standardised residual values from a linear regression of weight against head width (Schulte-Hostedde, Millar, & Hickling, 2001): a positive BCI is indicative of a better condition and greater energy reserves (Schulte-Hostedde et al., 2001).

Bank vole recapture procedure

Eighty-four bank voles were returned to their original trapping site (CL *n*=48; CH *n*=36; Figure 1b), with 1-4 males released at all but six locations where 5-9 males were released (Table S1). After nearly 5 weeks (mean=35.9 days, range=16-52 days), we recaptured 43 males (identified via the unique tag number, CL *n*=23; CH *n*=20). The recapture rate in both CL (48%) and CH (55%) areas was similar to CMR studies of wild bank voles elsewhere (Voutilainen, Kallio, Niemimaa, Vapalahti, & Henttonen, 2016). Not all recaptured animals provided sufficient faecal material within 2 hours of monitoring, leaving 56 samples from 28 individuals (CL1 *n*=3, CL2 *n*=13; CH1 *n*=8, CH2 *n*=4) for analysis. Animals were humanely euthanized to collect TLD dosimeters and tissues for other analyses. All procedures were performed in accordance with legal requirements and regulations from the Ukrainian authorities (957-i/16/05/2016), and the Animal Experiment Board in Finland (ESAVI/7256/04.10.07/2014). The samples were transported to Finland for research purposes based on the import permission from the Evira (3679/0460/2016).

DNA extraction, amplification, and sequencing

Total DNA was extracted from ~0.1 g of faecal material using a PowerFecal DNA Isolation kit following the manufacturer's instructions (MO BIO Laboratories, Inc.). Potential contamination of samples was limited following guidelines for sequence-based analyses of microbial communities (Eisenhofer et al., 2019; Salter et al., 2014). The DNA extractions were performed in a dedicated laboratory space under a laminar flow hood using aseptic techniques (surface sterilisation, use of sterile plasticware, and use of aerosol barrier tips for pipetting). In these settings, we did not use 'blank' water controls at the DNA extraction step. While the potential trace contamination derived from reagents is a larger problem in low microbial biomass microbiome studies compared to high microbial biomass microbiome studies (Eisenhofer et al., 2019), here the DNA was extracted from high microbial biomass faecal samples. And yet, to minimise effect of potential contaminants on results, we used the same DNA extraction kit batch for all the samples. Faecal microbiome samples were processed using the Earth Microbiome Project protocol to amplify the V4 region of the 16S ribosomal RNA (rRNA) gene using the original 515F/806R primers (Caporaso et al., 2012; Thompson et al., 2017). To control for potential contamination derived from reagents or the wider laboratory environment, negative controls were included during library preparation work at the Beijing Genomics Institute (BGI, www.bgi.com/global/). Libraries were sequenced on an Illumina MiSeq platform at BGI to provide 250 bp paired-end reads. The negative controls were not sequenced as they did not generate any PCR product. To avoid potential batch effects and systematic bias, samples from each treatment group were processed (storage, transportation, DNA extraction, and sequencing) at random.

Read data processing

Read data were de-multiplexed, and adapters and primers were removed by BGI. To resolve amplicon sequence variants (ASVs), read data were truncated at the 3' end to remove low

quality base calls (<Q25, only reverse reads were truncated at 166 bp) and de-noised in paired-end mode using DADA2 implemented by QIIME2 v.2018.2 (Bolyen et al., 2019; Callahan et al., 2016). The Greengenes v.13_8 16S rRNA gene sequences trimmed to the V4 region bound by the 515F/806R primer pair, and clustered at 99% identity, were used to pre-train the naïve Bayes classifier to assign taxonomy (Bokulich et al., 2018). Low-abundance (frequency <10 across all samples) features were removed. These steps left 1,448,198 sequences (14,981–40,855 sequences/sample) and 2,285 out of the total 2,905 ASVs. The data were rarefied at even 14,981 sequences/sample and, unless otherwise stated, this normalised feature-table was used for subsequent analyses to avoid biases caused by variation in sequencing depth among samples (Weiss et al., 2017)

Stable isotope analysis

Stable isotope analysis (SIA) was used to examine potential variation in bank vole diet (Calandra et al., 2015) (Figure 1b). We analysed stable isotope ratios of nitrogen (noted $\delta^{15}N$) and carbon ($\delta^{13}C$) from the fur of animals at first capture as its slow isotopic turnover rate reflects the isotopic signal of the diet during the 1-2 months prior to capture (Kurle, Koch, Tershy, & Croll, 2014). We analysed livers of recaptured animals, as half-lives of stable isotopes in liver tissue range from 2.8 to 7.7 days for $\delta^{13}C$ and between 3.6 and 15.1 days for $\delta^{15}N$ (Robb, Woodborne, de Bruin, Medger, & Bennett, 2015). Hence, the fur stable isotope data reflect bank vole diet in early spring (prior to first capture), whereas the 16-52 day interval between captures allows the liver to indicate the dietary preferences of bank voles during the CMR study, in early summer. Putative food items (n=136 samples of herbaceous and woody plants, insects, fungi, mosses, and lichens) (Butet & Delettre, 2011) were sampled from the dominant species present at trapping sites (see Supporting Information for methods).

Absorbed radiation doses

We used individual-level dosimetry to estimate external and internal (^{137}Cs burden) radiation exposure in sampled bank voles (see Supporting Information for methods). Between captures, bank voles inhabiting contaminated CH areas received significantly higher (mean=43.07 mGy) external radiation doses than did animals from CL (mean=0.33 mGy) ($p<0.05$, Wilcoxon rank-sum test; Table S1). Notably, the external dose of bank voles measured with TLDs was significantly, positively correlated with the doses estimated from the ambient radiation dose rates measured using hand-held Geiger counter at the bank vole trapping sites ($r=0.75$, $p<0.05$, Spearman's correlation). Thus, the external radiation dose absorbed by voles inhabiting the CEZ can be predicted from the ambient radiation dose rate at the bank vole trapping location. Similarly, bank voles inhabiting CH were exposed to significantly more (mean=25.6 mGy) internal radiation during the time spent in the field than animals from CL (mean=0.08 mGy) ($p<0.05$, Wilcoxon rank-sum test; Table S1). Hence, our dosimetry data indicate that bank voles inhabiting CH, but not CL, areas are chronically exposed to significant radiation doses derived from both external (inhabiting the area) and internal (ingesting contaminated food, water, soil particles) sources.

Statistical analyses

Statistical analyses were performed using R v.3.5.0 (R Core Team, 2018) unless otherwise stated. Pairwise comparisons were completed using either the Wilcoxon rank-sum, signed-rank (for paired observations) tests, or the Kruskal-Wallis tests when more than two groups were compared. We used a Benjamini-Hochberg false discovery rate (FDR) correction for multiple testing when appropriate. Differences in beta-diversity between samples were visualized by Principal Coordinates Analysis (PCoA), based on the Bray-Curtis dissimilarity, Jaccard similarity index, unweighted and weighted UniFrac distances (Jaccard, 1908;

Lozupone, Hamady, Kelley, & Knight, 2007; Lozupone & Knight, 2005; Sørensen, 1948). Statistical significance of sample grouping was assessed by a permutational multivariate analysis of variance (PERMANOVA, 999 permutations) using the ADONIS function in R package VEGAN implemented in QIIME2 V.2019.4 (Bolyen et al., 2019; Oksanen et al., 2018). In addition to univariate models including either treatment or CMR study categories, we have constructed separate models for the first and second microbiota observations to explore the influence of other variables and ensure sample independence (Anderson, 2001). Since tests in ADONIS are sequential, categorical variables were fitted first, followed by linear terms, starting from those being generally significantly different between treatments. Thus, the models were constructed for the first microbiota observations including treatment, total radiation dose, body condition index, fur $\delta^{13}C$, and fur $\delta^{15}N$; and for the second observations including treatment, total radiation dose, body condition index, liver $\delta^{13}C$, liver $\delta^{15}N$, and the time animals spent in the lab and in the field. We used a PERMDISP test in QIIME2 to assess potential differences in group dispersion between treatments (Anderson, 2001). Significant differences in alpha diversity (measured as number of observed ASVs and the Shannon index (Shannon & Weaver, 1949)) between the study areas and within each study area over time were identified using Kruskal–Wallis tests implemented in the DUNN.TEST package (Dinno, 2017).

Differential abundance testing was performed using the permuted mean difference tests with 1000 permutations and discrete FDR (dsFDR) correction at alpha 0.1 (Jiang et al., 2017). We also used balances in GNEISS, a more sensitive method for detecting community-wide perturbations based on environmental parameters (Morton et al., 2017). We constructed trees to define partitions of features that co-occur (*i.e.* balances), based on the (1) unsupervised hierarchical clustering (Ward's clustering) and (2) gradient clustering using the total absorbed

radiation dose (from both internal and external radiation exposures) for each bank vole individual. Further details are provided in Supporting Information.

Results

Exposure to radiation impacts composition, but not alpha diversity of bank vole gut microbiota

The gut microbiota of bank voles was comprised of 10 bacterial phyla, dominated by the Firmicutes (47% of all reads), Bacteroidetes (36%), and Spirochaetes (9%; see Table S2). We found no significant difference in the bank vole gut microbiome alpha diversity (number of ASVs, Shannon Index) between or within radionuclide contamination treatments across time (p>0.05, Kruskal–Wallis test; Figure S1, Table S3). Forty-four ASVs differed significantly in abundance between contaminated (CH) and uncontaminated (CL) areas at first capture (p<0.05, permutation test with dsFDR correction; Table S4), with a marked enrichment of members of the *S24-7* family (Bacteroidetes) in samples from CL and an increase in ASVs assigned to *Ruminococcaceae*, *Lachnospiraceae* (Firmicutes), and *Desulfovibrionaceae* (Proteobacteria) families in CH samples (Figure 2). Hence, exposure to environmental radionuclides in early summer is associated with the altered composition of the bank vole gut microbiota.

The 'temporal core' gut microbiota, defined as ASVs present in >90% individuals per treatment over time (Risely, 2020), comprised 8 and 18 ASVs in samples from CL and CH areas respectively (Table S5). Bank voles from CH had more than twice the number of ASVs in their temporal core gut microbiota than did animals from CL. This was not related to alpha diversity *per se*, as neither of the alpha diversity measures differed significantly between

treatments (p>0.05, Kruskal–Wallis test; Figure S1, Table S3). Thus, bank voles from CH maintain larger temporal core gut microbiota as compared with animals from CL.

Bank voles exposed to radiation exhibit distinct gut microbiota profiles

Bank vole gut microbiota significantly differed among radionuclide contamination treatments (p<0.05, PERMANOVA; Table S6). Thus, at both captures, CL samples were separated from CH principally along the first PCoA axis (Figure 3a for Bray-Curtis, see also Figure S2 for other distance metrics). While exposure to environmental radionuclides was a dominant predictor of bank vole gut microbiota structure, within CH and CL the total radiation doses explained little (3-4%) additional variation in the gut microbiota profiles (Table S6). These patterns were largely consistent across three out of four beta-diversity metrics (*cf*. weighted UniFrac; Figure 3a and Figure S2, Table S6). Due to the lack of large phylogenetic turnover, differences between CH and CL samples were less apparent when using phylogenetic metrics (Lozupone & Knight, 2005). We found that differences in gut microbiota profiles between CH and CL voles resulted not only from shifts in group centroids, but also from significant differences in dispersion between treatments (p<0.05, PERMDISP; Table S6). These effects were clearly driven by temporal variation in the gut microbiota of bank voles inhabiting CL areas (Figure 3a).

Temporally stable gut microbiota in bank voles exposed to radiation

Our capture-mark-recapture (CMR) study revealed that radiation exposure was associated with a contrast in the temporal stability of bank vole gut microbiota. Thus, by the second capture, the early summer difference in the community composition between treatments had narrowed, principally because of changes in the gut microbiota of bank voles inhabiting CL areas: There was a significant decrease in abundance of members of the *S24-7* family (>10%

reduction in relative abundance) and an increase in taxa from the *Ruminococcaceae* and *Lachnospiraceae* (p<0.05, permutation test with dsFDR correction; Figure 2, Table S4). Importance of such concomitant rearrangements for the differences in the gut microbiome profiles was verified when the *S24-7* taxon was removed from consideration (Figure S3). By contrast, the gut microbiota of bank voles inhabiting areas contaminated by radionuclides exhibit temporal stability with none of the ASVs showing a significant temporal change in abundance (p>0.05, permutation test with dsFDR correction). Hence, bank voles inhabiting CL, but not CH, areas had significant temporal changes in gut microbiota beta-diversity (p<0.05, for Bray-Curtis, Jaccard similarity index and unweighted UniFrac; but p>0.05 for weighted UniFrac PERMANOVA; Table S6). Moreover, consistent across trapping sites replicates, only samples from CL areas characterised by significant gut microbiota divergence over time (CL W=9, p<0.01; CH W=30, p=0.48, Wilcoxon signed-rank tests; measured as pairwise difference in the Bray-Curtis PCoA axis 1, Figure 3b).

Using the differentially-represented taxa (log-ratios in GNEISS (Morton et al., 2017)) associated with either CH (ASVs from the Clostridiales order) or CL (mostly ASVs from the *S24-7* family) samples, we could readily differentiate between the samples from CH and CL areas collected at both time points (F-statistic=32, p=6x10^{-6} at first capture; F-statistic=15.83, p=0.0004 at second capture), and between samples from voles at first and second captures within CL, but not between sampling times within CH areas (F-statistic=24.43, p<0.0001 and F-statistic=24.43, p=0.98, respectively; Figure 3c; hierarchy-based balance, Table S7). Moreover, using a radiation dose-based balance, we could predict the total radiation dose absorbed by each bank vole individual between captures (r=0.73, p=1.5x10^{-10}, Pearson correlation; Figure 3d), with consistent results obtained when either the external or internal

radiation dose estimates were used to define the data partitions (balances) (Table S7). Hence, balance analysis reinforces the idea that radiation exposure constrains the gut microbiota.

Temporal changes in gut microbiota were not associated with the time CH animals spent in the field between captures (r=-0.09, p=0.82 for pairwise Bray-Curtis dissimilarity, Pearson correlation), but there was a qualitatively smaller temporal change in gut microbiota structure within individuals as the total radiation dose increased (r=0.14, p=0.12 for pairwise Bray-Curtis dissimilarity, Pearson correlation). While radiation dose was inevitably a poor predictor of the level of temporal change in gut microbiota of individuals in CL areas due to absence of radionuclide contamination, the time between captures was positively and significantly associated with the magnitude of change in the gut microbiome community structure (r=0.35, p>0.009 for pairwise Bray-Curtis dissimilarity, Pearson correlation).

Bank vole diet and body condition do not explain differential stability in gut microbiota structure

Mean fur $\delta^{13}C$ was significantly higher in the CH voles compared with CL animals (W=162, p=0.002, Wilcoxon rank-sum test), whereas mean fur $\delta^{15}N$ did not significantly differ between treatments (W=74, p=0.32, Wilcoxon rank-sum test; Figure 4a). Differences in fur $\delta^{13}C$ imply that, in early spring (before the start of the CMR study, Figure 1b) the diet of bank voles from CH and CL areas potentially differed in dietary sources of carbon, *i.e.* woody and herbaceous plants. However, while according to the fur SIA data average diets varied between CH and CL along the carbon axis (see also Figure S4 for differences in inter-tissue variability in $\delta^{13}C$ between CH and CL areas), the amount of dietary variation of both dietary carbon and nitrogen was similar between treatments (F=0.48, p=0.49 for $\delta^{13}C$; F=0.72, p=0.40 for $\delta^{15}N$, Levene's test). Thus, neither fur $\delta^{13}C$ nor $\delta^{15}N$ isotopes provided

statistically significant associations with the gut microbiome structure (p>0.05, PERMANOVA; Table S6)

In liver tissue, there was no significant difference in $\delta^{13}C$ or $\delta^{15}N$ between treatments ($\delta^{13}C$ W=82, p=0.53 and $\delta^{15}N$ W=69, p=0.21, Wilcoxon rank-sum test; Figure 4a), with neither $\delta^{13}C$ nor $\delta^{15}N$ isotopes in bank vole livers having a significant explanatory effect on gut microbiota structure based on most beta-diversity metrics (p>0.05, PERMANOVA; but see an effect of $\delta^{15}N$ on the Jaccard index; Table S6). There were no significant differences in variance between CH and CL voles in the $\delta^{13}C$ (F=0.66, p=0.42, Levene's test) or $\delta^{15}N$ isotopes values (F=0.56, p=0.45, Levene's test). Thus, the isotopic signal from liver tissue indicates that there is no major difference in bank vole summer diet between radionuclide contamination treatments.

The body condition index (BCI) differed significantly between treatments, with animals inhabiting CL areas having a higher BCI compared with CH voles (W=153, p=0.007, and W=141, p=0.03 at first and second captures, respectively, Wilcoxon rank-sum tests; Figure 4b). In both areas, the BCI changed little with time (χ^2=12.08, p=0.41 and p=0.40, for CH and CL respectively, Kruskal–Wallis test), and there was a significant negative correlation between BCI and total radiation dose (r=-0.53, p=0.004 at first capture; r=-0.37, p=0.03 at second capture, Spearman's correlation; Figure 4c). Such differences between treatments in BCI, however did not explain variation in gut microbiome beta-diversity (p>0.05, PERMANOVA; Table S6).

Discussion

Gut microbiota are an important component of animal host health and it is therefore important to recognise dysbiosis. Using capture-mark-recapture (CMR) of wild bank voles inhabiting areas that differed in radionuclide contamination (Figure 1), we sought to test key predictions of the *Anna Karenina principle* (AKP) for animal microbiomes that postulates that exposure to stress destabilises the microbiota, leading to a stochastic increase in inter-individual differences in microbiome profiles, and temporal changes in community composition (Zaneveld et al., 2017). Counter to our expectations, exposure to radionuclides is associated with a constrained gut microbiota community in bank voles, whereas animals inhabiting uncontaminated areas harbour a variable and temporally dynamic gut microbiota.

Environmental stress and bank vole gut microbiota stability

Bank voles inhabiting contaminated areas within the CEZ experience radiation from external and internal sources. For reference, the average total radiation dose absorbed by bank voles from CH areas between captures (mean=68.66 mGy) is equivalent to about 450 chest radiography scans (X-rays) (Brenner & Hall, 2007), whereas over a similar course of five weeks, animals from CL received a dose equivalent to less than three chest X-rays (mean=0.40 mGy). We hypothesised that such chronic radiation exposure induces stress in CH voles (here defined as a condition of failure to control a critical variable (Del Giudice et al., 2018)). Evidence that bank voles inhabiting CH, but not CL areas, experience stress associated with chronic radiation exposure is derived from the lower BCI score (Figure 4b,c) and other studies showing altered cellular homeostasis (Kesäniemi, Lavrinienko, et al., 2019). With this in mind, it is perhaps not surprising that the gut microbiota of bank voles exposed to environmental radionuclides differs from the gut microbiota of animals in uncontaminated areas (Figure 2 and 3, see also (Lavrinienko, Mappes, et al., 2018)). Our data are consistent

with other studies that have found stress to shift the gut microbiota in animals and humans (Karl et al., 2018; Marin et al., 2017; Rocca et al., 2019; Zaneveld et al., 2017). However, temporal stability of the gut microbiota in bank voles exposed to radiation is somewhat unexpected and counter to predictions of the AKP (Figure 3; Zaneveld et al., 2017). Notably, high inter-individual difference in microbiome profiles is not some artefact of the habitats within the CEZ, as the gut microbiota of bank voles inhabiting CL areas are comparable with the gut microbiota of bank voles from areas outside the CEZ (elsewhere in Ukraine), that have no chance to become exposed to radionuclides (Lavrinienko, Mappes, et al., 2018; Lavrinienko, Tukalenko, et al., 2018). Moreover, in the absence of exposure to radiation, bank vole gut microbiota are apparently free to adopt a wider range of community configurations over time. High variability and a capacity for temporal changes in composition thus appear to reflect the typical, 'healthy' state of wild rodent gut microbiota rather than stress and dysiosis (Maurice et al., 2015; Ren et al., 2017). Thus, while environmental stress can alter microbial community composition, it does not necessarily induce stochastic changes or influence temporal stability in gut microbiome structure (Figure 3).

Constraints on microbiota may be driven by a strong environmental filter on the gut microbiota. For example, exposure to radionuclides might select for certain individual taxa, an effect referred to as the anti-AKP by Zaneveld et al. (2017). Such strong selection seems unlikely, as the CL and CH areas maintain similar alpha diversity (Figure S1, see also (Lavrinienko, Mappes, et al., 2018)). Notwithstanding the radiation dose, bacteria are expected to be capable of evolving resistance to chronic radiation exposure (Harris et al., 2009), and free-living prokaryotes isolated from the CEZ show signs of radioresistance (Hoyos-Hernandez et al., 2019; Ragon et al., 2011; Theodorakopoulos et al., 2017). Given that microbiota associated with animals are derived from the pool of microbes available in

the environment (Bletz et al., 2016) and influenced by host diet (Maurice et al., 2015; Ren et al., 2017), the altered gut microbiota of bank voles in contaminated areas may be a secondary consequence of a wider ecological impact of environmental radionuclides. The few differences in the composition of skin microbiota of bank voles inhabiting contaminated and uncontaminated areas within the CEZ (Lavrinienko, Tukalenko, et al., 2018), however, imply that environmental radionuclides *per se* have little influence on the pool of microbes in the external environment. Since the SIA fur data indicate some differences in bank vole diet between CL and CH before the first capture (in early spring) (Figure 4a, Figure S4), the composition of diet is a plausible reason for the association between radiation and bank vole gut microbiota profiles. Although, as the variance in SIA profiles does not differ between treatments, in contrast to microbiota beta-diversity (Figure 3a,b), it seems unlikely that dietary variation accounts for the different patterns of bank vole gut microbiota variability among contaminated and uncontaminated areas over time.

Healthy bank voles harbour dynamic gut microbiota in nature

Longitudinal sampling provides essential context to any definition of a 'healthy' microbiota, but is still rare in studies on wild animals. In the absence of environmental stress (radiation exposure), the gut microbiota of wild bank voles are varied and dynamic (Figure 3a). Temporal changes in the gut microbiota of wild animals is a natural response to seasonal changes in diet or physiology (*e.g.* as animals prepare for breeding season or wintering; Maurice et al., 2015; Ren et al., 2017; Sommer et al., 2016). The diet of bank voles is seasonally variable and dietary data from literature suggest that typically, bank voles respond to seasonal changes by considerable fluctuations in consumption of forbs, berries, invertebrates, lichens, and fungi (Calandra et al., 2015). With this in mind, our CMR study highlights two key features of wild animal gut microbiota: (1) a seasonal change of the gut

microbiota profiles in bank voles from CL that has a consistent directional change in composition (Figure 3b), despite deliberately heterogeneous sampling (Figure 1a), and (2) temporal stability and resilience of CH gut microbiota (Figure 3) under chronic exposure to radionuclides.

Estimates of accumulated radiation dose (TLD data) show that bank voles are relatively sedentary and during the period of CMR did not move much between uncontaminated and contaminated areas. The crucial implication of these data is that a more gradual exposure to radiation in CL animals does not explain the replacement of Bacteroidetes (*S24-7*) by Firmicutes (*Ruminococcaceae* and *Lachnospiraceae*) in CL (Figure 2). Rather, our SIA data at second capture (where liver samples do not differ in SIA profiles between treatments, Figure 4a) indicate some tendency for the diet of bank voles in CL habitats to become more similar to the diet of voles in CH areas. Alternatively, bank voles exposed to radionuclides have altered metabolism (Kesäniemi, Jernfors, et al., 2019). Given that metabolic changes and gut microbiome rearrangements in wild animals coincide with seasonal change (Sommer et al., 2016; Stevenson, Duddleston, & Buck, 2014), it is possible that the seasonal changes in CL microbiota reflects host control over gut microbes via a similar internal metabolic shift. Such an explanation is consistent with the observed reduction in the *S24-7* family (Figure 2, Table S4) that appears to be sensitive to variation in diet (Ormerod et al., 2016) and season (Stevenson et al., 2014) in other studies. In accordance with their distribution mostly in the gut of herbivorous and omnivorous animals (woodrat, bank vole, and mouse have the highest >70% prevalence across studied mammals (Lagkouvardos et al., 2019)), genome analysis revealed that *S24-7* (other proposed names are *Homeothermaceae* (Ormerod et al., 2016) and *Muribaculaceae* (Lagkouvardos et al., 2019)) are versatile with respect to complex carbohydrate degradation. Based on enzymatic capacities, the *S24-7* members were grouped

into the three trophic guilds: alpha-glucans (starch), complex plant cell wall glycans (hemicellulose and pectin), and host-derived glycans (Ormerod et al., 2016). Which guild is more common or whether these different guilds co-occur in bank vole gut microbiota is unknown. However, the overall carbohydrate-based trophic niche of the *S24-7* and high dependency on the diet-derived polysaccharides, highlight potential sensitivity of the *S24-7* to dietary or seasonal fluctuations (Lagkouvardos et al., 2019; Ormerod et al., 2016). In the context of the AKP, it is notable that temporal changes in gut microbiota of animals inhabiting CL are unlikely to be a dysbiotic response to some unrecognised stressor within CL, because this area is characterised by animals that have a high and stable BCI score (Figure 4b). Thus, seasonal changes in gut microbiota had little impact on energy balance. Our data are consistent with the idea that a variable gut microbiota is likely beneficial in nature (Alberdi et al., 2016), as gut microbiota plasticity provides the host with diverse metabolic pathways that can facilitate exploitation of resources in a changing environment.

Given that the gut microbiota can influence host fitness (Alberdi et al., 2016), co-evolution between bank voles and their gut microbes in CH areas might select for a distinct gut microbiota profile that provides beneficial services under conditions of chronic radiation exposure. For example, genera within the *Ruminococcaceae* and *Lachnospiraceae* families that dominate the gut microbiota of CH voles could provide direct benefit to the host in the form of short-chain fatty acids (SCFAs) (Rajilić-Stojanović et al., 2014), metabolites with vast physiological effects that can boost host health (Gentile & Weir, 2018; Hamer et al., 2007). The production of SCFAs by the gut microbiota, however, is not limited to these two bacterial families. Conversely, given the multiple lines of evidence indicating that bank voles exposed to radionuclides within the CEZ are stressed (Jernfors et al., 2018; Kesäniemi, Lavrinienko, et al., 2019; Lehmann et al., 2016; Mappes et al., 2019), including the negative

association between BCI and total radiation dose (Figure 4c), a constraint to gut microbiota would appear more a sign of dysbiosis. It is interesting that bank voles inhabiting the CEZ have altered metabolism and immunity profiles (Kesäniemi, Jernfors, et al., 2019), the two systems (*i.e.* immunity and metabolism) that play central roles in microbiome stability (Gentile & Weir, 2018; Hooper et al., 2012). Therefore, it is plausible that the gut microbiome of bank voles inhabiting radioactively contaminated areas is constrained by the physiology of a stressed host and this prevents a dynamic response by the gut microbiota to natural spatio-temporal variation in resources. Further experiments are needed to determine whether environmental radionuclides impose a direct (on host or bacteria) or indirect (on environment and available diet) impact on bank vole gut microbiota.

Conclusions

The AKP for animal microbiomes provides a much-needed framework to understand effects of environmental stressors that are now commonplace (Rocca et al., 2019; Trevelline et al., 2019), but have unknown consequences for most wildlife microbiota. Such a predictive theory for 'stressed' microbiomes is clearly complicated by the fact that outcomes of environmental stress are context-dependent, with stressors varying in mode of action, severity, frequency, and duration (Karl et al., 2018; Rocca et al., 2019). Hence, longitudinal studies are increasingly critical for documenting effects of various stressors on microbiome stability in contrast to natural dynamics to further develop the AKP concept for wild animal microbiomes.

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Authors' contributions

AL, PW, and TM designed research; AL, ET, JK, KK, SM, ZB, TAM, GM, TM, PW collected the data; AL, analysed data and wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data Accessibility Statement

Sequence data have been uploaded to the European Bioinformatics Institute (EBI) database using QIITA (project ID: 12325; EBI accession number: ERP114357). The datasets (QIIME2 files), code, and metadata supporting this article are available on this URL: https://github.com/alavrinienko/chern-cmr-voles, with the final version also archived in Zenodo with a DOI http://doi.org/10.5281/zenodo.3993420.

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Figures

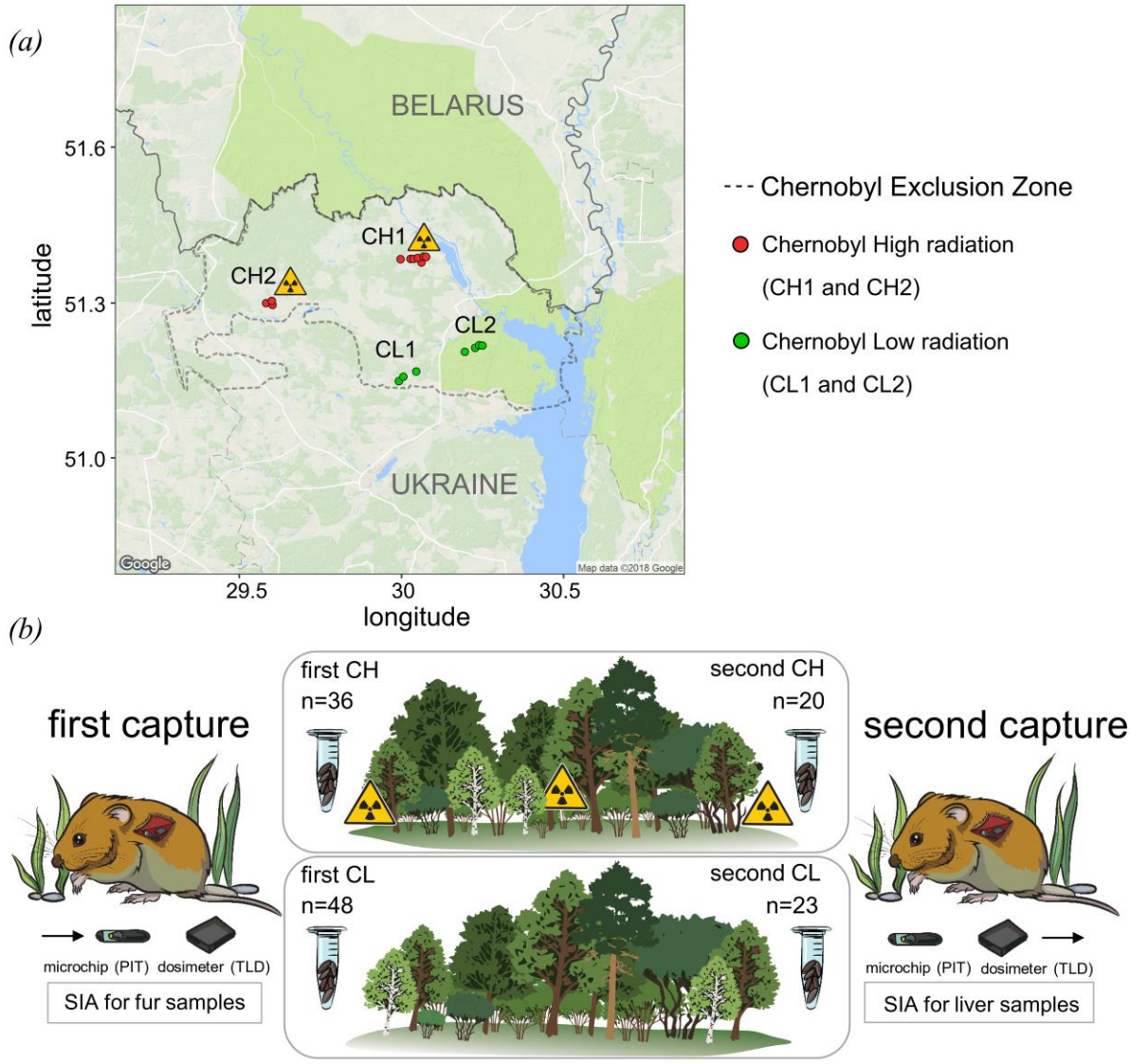

Figure 1. A capture-mark-recapture (CMR) survey of wild bank voles (*Myodes glareolus*) inhabiting areas that contrast in levels of radionuclide contamination. *(a)* Map of the study areas with bank vole trapping sites shown by points. Replicate sites within each area (*e.g.* CH1-2, CL1-2) are shown, with areas contaminated (CH) and uncontaminated (CL) with radionuclides within the Chernobyl Exclusion Zone, Ukraine. Figure was created using GGMAP v.2.6.1 package in R. *(b)* Schematic representation of the capture-mark-recapture study design. The gut microbiota were analysed at both captures, additionally bank voles diet

(using stable isotope analysis, SIA) and radiation exposure (using implanted dosimeters and γ-spectrometry) during the CMR study were examined.

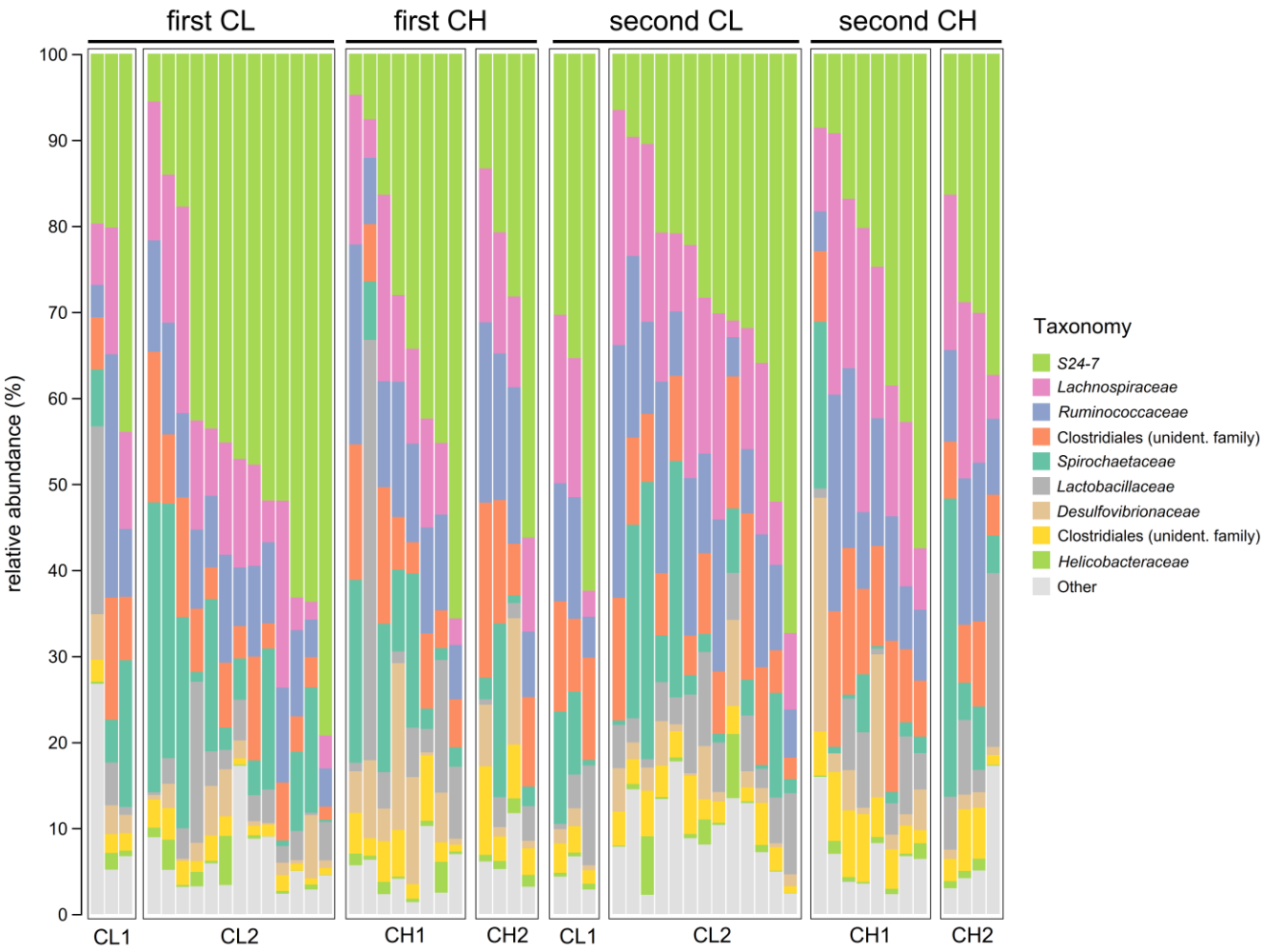

Figure 2. Relative abundance of bacterial taxa (at the family level) in the gut microbiota of bank voles inhabiting areas that contrast in levels of radionuclide contamination. The community composition for individual samples is shown (taxa with the relative abundance of less than 1%, collectively refer to as 'Other'). Samples from the first and second captures of bank voles within each contaminated (CH) and uncontaminated (CL) trapping sites replicates (*e.g.* CL1-2, CH1-2) within the Chernobyl Exclusion Zone are grouped separately.

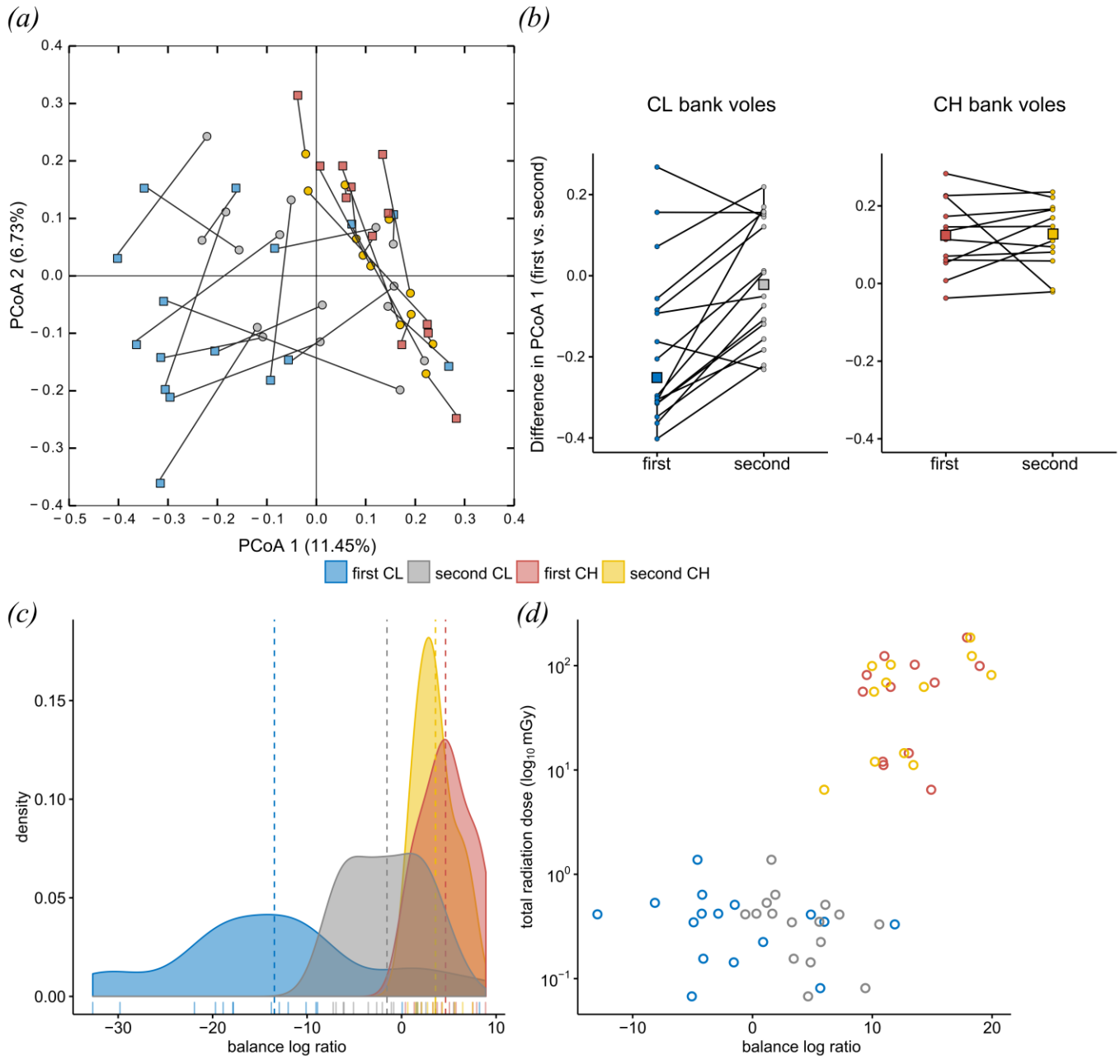

Figure 3. Differences in the gut microbiota profiles of bank voles inhabiting areas that contrast in levels of radionuclide contamination. *(a)* PCoA based on the Bray–Curtis dissimilarity between the gut microbiota profiles of bank voles inhabiting areas contaminated (CH) and uncontaminated (CL) with radionuclides within the Chernobyl Exclusion Zone, Ukraine. Each point represents a single sample, shape indicate paired first (square) and second (circle) samples from a recaptured individual that are connected by a solid line. *(b)* Differences in the Bray-Curtis dissimilarity PCoA axis 1 among study areas over time.

Samples from same individual are connected by a solid line; small squares denote median values per group (p<0.01 for CL, but p=0.48 for CH, Wilcoxon signed-rank tests). The balance trees analysis *(c* and *d)* differentiating samples from CH and CL areas based on differentially represented taxa (log ratios; p<0.001 at both captures). The data partitions (balances) defined by *(c)* unsupervised hierarchical-clustering (density plot height display where values are concentrated, whereas its shape display samples distribution; dotted lines indicate mean values) and *(d)* gradient-clustering using the total radiation dose absorbed by each bank vole individual.

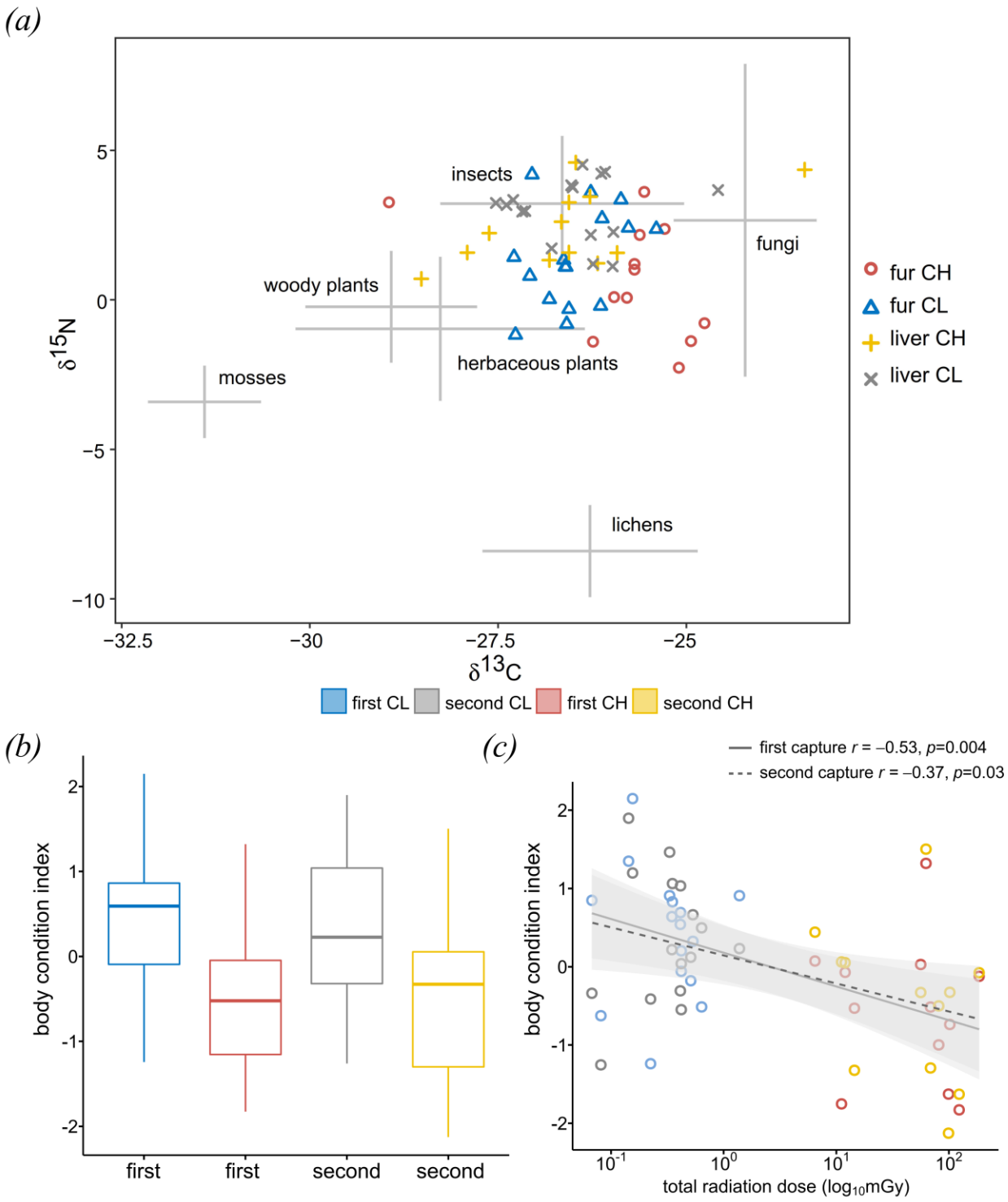

Figure 4. Variation in dietary profiles (estimated using stable isotope analysis) and body condition index in bank voles inhabiting areas that contrast in levels of radionuclide contamination. *(a)* Carbon ($\delta^{13}C$) vs. nitrogen ($\delta^{15}N$) isotopic values for the fur and liver tissues of bank voles inhabiting areas contaminated (CH) and uncontaminated (CL) with

radionuclides within the Chernobyl Exclusion Zone, Ukraine (see Supporting Information for methods; only mean fur $\delta^{13}C$ comparisons were significant, $p<0.01$, Wilcoxon rank-sum test). Dietary sources are presented with means and SD. *(b)* Differences in body condition index (BCI) score between bank voles inhabiting CH and CL areas ($p<0.05$ between CH and CL at both captures, Wilcoxon rank-sum test; comparisons within CH and CL over time were non-significant, Kruskal-Wallis test). *(c)* Correlation between BCI and total radiation dose absorbed by each bank vole individual ($p<0.05$ at both captures, Spearman's correlation).