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# Spatial and temporal characteristics of bacterial parasite communities in outbreaking fossorial water vole (*Arvicola terrestris*) populations: static uniformity or dynamic heterogeneity?

Petra Villette

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Petra Villette. Spatial and temporal characteristics of bacterial parasite communities in outbreaking fossorial water vole (*Arvicola terrestris*) populations: static uniformity or dynamic heterogeneity?. Agricultural sciences. Université Bourgogne Franche-Comté, 2018. English. NNT : 2018UBFCD056 . tel-01931281

**HAL Id: tel-01931281**

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**THESE DE DOCTORAT DE L'ETABLISSEMENT  
UNIVERSITE BOURGOGNE FRANCHE-COMTE  
PREPAREE A UMR 6249 CHRONO-ENVIRONNEMENT**

Ecole Doctoral n°554  
Environnement-Santé

Doctorat de Biologie

Par  
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Spatial and Temporal Characteristics of Bacterial Parasite Communities in  
Outbreking Fossorial Water Vole (*Arvicola terrestris*) Populations: Static Uniformity  
or Dynamic Heterogeneity?

Thèse présentée et soutenue à Besançon le 28 juin 2018

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# Abstract

Multiannual population fluctuations in rodents have fascinated scientists for over 100 years, and have proven remarkably resistant to explanation. The fossorial water vole *Arvicola terrestris* in the region of Franche-Comté is one such species that exhibits these multiannual outbreaks every 5-8 years (mean 6 years), with peak densities exceeding 200 individuals/ha, and reaching up to > 600 individuals/ha. At such high densities, voles cause significant agricultural losses and also poses a risk to human health as a pathogen reservoir. Peak densities can persist locally at the commune scale (~25 km<sup>2</sup>) for up to 3 years, and currently there is no method for predicting when the decline will occur. Current hypotheses regarding rodent population outbreaks do not explain the timing of the decline phase in *A. terrestris* in this region; predators alone do not appear to be capable of instigating the crash, and declines can occur even when there is abundant food. Pathogens and parasites are one hypothesis that has not received much attention in this system, and nothing is known about the bacterial parasites hosted by this rodent. Bacterial parasites are likely an important component of the parasite community in this species, may play a role in the decline, and may also be a source of tools for predicting when the decline will occur.

We present here the results of three years of work investigating bacterial parasites in *A. terrestris* collected from five communes with increasing or highly abundant *A. terrestris* populations in the Jura and Doubs departments of Franche-Comté, France. Using metabarcoding and high-throughput sequencing techniques, we have attempted to characterize the spatial and temporal heterogeneity of the bacterial parasite communities of this rodent at several scales. We conducted a pilot study to assess the heterogeneity in bacterial communities of different organs within individual hosts, and found that the heart, liver, lungs, spleen and kidneys host different bacteria within an individual animal, and that all five organs are necessary for this coverage of the bacterial parasite community hosted in these individuals.

Using animals collected in spring and autumn 2014-2016, we measured individual host (infracommunity) and host population (component community) bacterial parasite richness as well as pairwise similarities of bacterial communities at these two scales. We found that infracommunity richness was best predicted by extrinsic factors (location and date), and that while infracommunity composition is highly heterogeneous even within populations, infracommunity similarity is significantly (if weakly) correlated with geographic distance. We determined that component community richness is best predicted by current local abundance, and that geographic distances between sites and component community similarity are not correlated.

To assess long-term temporal heterogeneity in bacterial community composition, we compared the spleen bacterial parasite communities of *A. terrestris* collected from three of our study sites in 2003-2005 to splenic communities collected in 2015-2016; 11 OTUs of the 22 detected in 2003-2005 were detected in 2015-2016, and of the 11 not detected in the recently collected spleens, 6 were detected in other organs of the animals tested. We used the IndVal approach to identify potential indicators of the late high-abundance phase as a potential management tool for predicting the timing of the decline; however, only one of the three bacteria we identified as an indicator of the 2003-2005 late high-abundance phase appear in spleens collected in 2015-2016.

We have demonstrated that high-throughput sequencing and metabarcoding techniques are useful for identifying bacterial parasites in rodent hosts if appropriate sampling methods are employed, and have formed a foundation upon which we can build further investigations into the bacterial parasite community of *A. terrestris* in this region. Additional samples collected in 2017-2018 during the decline and low density phases will likely provide more insights.

## Résumé

Les fluctuations pluriannuelles de population chez les rongeurs fascinent les scientifiques depuis plus de 100 ans et se sont révélées remarquablement résistantes à leur explication. En Franche-Comté, le campagnol terrestre *Arvicola terrestris* est l'une de ces espèces qui présente ces pullulations pluriannuelles tous les 5-8 ans (moyenne 6 ans), avec des densités maximales supérieures à 200 individus / ha, et jusqu'à 600 individus / hectare. À de telles densités, il cause des pertes agricoles importantes et présente également un risque pour la santé humaine, comme réservoir de pathogènes. Les hautes densités, lors des pics de pullulation, peuvent persister localement à l'échelle de la commune pendant une période allant jusqu'à trois ans, et il n'existe actuellement aucune méthode permettant de prédire quand le déclin se produira. Les hypothèses actuelles concernant les pullulations de populations de rongeurs n'expliquent pas le timing de la phase de déclin chez *Arvicola terrestris* dans cette région; les prédateurs ne semblent pas être à eux seuls capables de provoquer le déclin, et des déclins peuvent se produire même lorsque la nourriture est disponible en abondance. Les pathogènes et les parasites sont une hypothèse qui n'a pas reçu beaucoup d'attention dans ce système, et on ne sait rien sur les parasites bactériens hébergés par ce rongeur. Les parasites bactériens sont probablement une composante importante de la communauté parasitaire chez cette espèce, peuvent jouer un rôle dans le déclin et peuvent également être une source d'outils pour prédire quand le déclin se produira.

Nous présentons ici les résultats de trois années de travaux sur les communautés de parasites bactériens d'*Arvicola terrestris* récoltés dans cinq communes à populations d'*Arvicola terrestris* en croissance ou à haute densité dans les départements du Jura et du Doubs en Franche-Comté. En utilisant des techniques de métabarcoding et de séquençage à haut débit, nous avons tenté de caractériser l'hétérogénéité spatiale et temporelle des communautés de parasites bactériens de ce rongeur à plusieurs échelles. Nous avons mené une étude pilote pour évaluer l'hétérogénéité des assemblages bactériens de différents organes chez les individus. Nous avons constaté que le cœur, le foie, les poumons, la rate et les reins abritent différentes bactéries au sein d'un même animal, et que les 5 organes sont nécessaires pour couvrir la communauté des parasites bactériens hébergés dans ces individus.

En utilisant les animaux capturés au printemps et à l'automne de 2014-2016, nous avons mesuré la richesse parasitaire bactérienne individuelle (infracommunity) et de la population hôte (component community) ainsi que les similitudes de paires de communautés bactériennes à ces deux échelles. Nous avons trouvé que la richesse infracommunautaire était mieux prédite par des facteurs extrinsèques (localisation et date), et que même si la composition infracommunautaire est hautement hétérogène au sein des populations, la similarité infracommunautaire est significativement (mais

faiblement) corrélée avec la distance géographique. Nous avons trouvé que l'abondance locale actuelle prédisait le mieux la richesse de la communauté, et que la distance géographique entre les sites et la similarité de la component community ne sont pas corrélées.

Pour évaluer l'hétérogénéité temporelle à long terme de la composition de la communauté bactérienne, nous avons comparé les communautés de parasites bactériens spléniques d'*Arvicola terrestris* recueillies dans trois de nos sites d'étude en 2003-2005 aux communautés spléniques collectées en 2015-2016; 11 OTU des 22 détectés en 2003-2005 ont été détectés en 2015-2016, et parmi les 11 non détectés dans les échantillons de rate les plus récents, 6 ont été détectés dans d'autres organes des animaux testés. Nous avons utilisé l'approche IndVal pour identifier les indicateurs potentiels de la phase de haute abondance tardive en tant qu'outil de gestion potentiel pour prédire le moment du déclin; cependant, une seule des trois bactéries que nous avons identifiées comme un indicateur de la phase de haute abondance tardive de 2003 à 2005 apparaît dans les rates prélevées en 2015-2016.

Nous avons démontré que les techniques de séquençage et de métabarcoding à haut débit sont utiles pour identifier les parasites bactériens chez les rongeurs hôtes si des méthodes d'échantillonnage appropriées sont utilisées et nous avons posé les bases permettant d'approfondir les recherches sur la communauté parasitaire bactérienne d'*Arvicola terrestris* dans cette région. Des échantillons supplémentaires recueillis en 2017-2018 pendant la phase de déclin et de basse densité fourniront bientôt plus d'informations.

## Acknowledgements

I would like to thank my three supervisors, Patrick Giraudoux, Jean-François Cosson and Eve Afonso for their direction, support, constructive criticism, assistance in the field and the lab, and coffee throughout this PhD; it's been a wild ride and I'm grateful to have had the three of you there every step of the way. I would also like to thank the members of my thesis committee, Gwenaël Vour'c and Serge Morand, for their feedback and support throughout the project, thank you for such great committee meetings!

The field work underpinning this thesis would not have been possible without the help of many people, most especially Geoffroy Couval and Aurélien Levret of the FREDON-FC (Fédération Régional de Défense controles Organismes Nuisibles de Franche-Comté). I would also like to thank additional FREDON-FC personnel whose assistance in the field was very much appreciated: Julie Montas, Herminie Piernavieja, Aurélie Guislain-Vitek, Marilyne Fontanier, Bérangère Foscarin, and Nicolas Bassin. Thibault Moulin, Virgile Baudrot, Javier Fernandez-de-Simon, Amaël Dupaix, Dominique Rieffel, Vincent Driget, and José Gonzalo Zapata Carbonell of the Laboratoire Chrono-Environnement were kind enough to volunteer their time and come help in the field, thank you!. Anne-Claude Goydadin-Gabiot assisted me with lab work on several occasions, for which I am very grateful.

A big thank-you goes to the staff of the Centre de Biologie pour la Gestion des Populations in Montpellier for hosting me for five weeks in 2015, especially Caroline Tatard for her excellent training in the lab and assistance in completing our first sequencing run, and Max Galan for PCR quantification of the sequencing libraries, and sequencing analysis and taxonomic classification of all of our sequencing runs; thank you so much for all of your work and your patience when answering my questions.

Historical samples were collected under the direction of Dr. Nathalie Charbonnel of the Centre de Biologie pour la Gestion des Populations in Montpellier; thank you for sharing them and their associated data with me. Thank you to Jean-Baptiste Pons for training Aurélien and I in field dissection.

This research has been supported financially by the Ministère de l'Alimentation de l'Agriculture et de la Pêche in conjunction with the Chambre de Agriculture Jura, the Chambre Interdépartementale d'Agriculture Doubs-Territoire de Belfort, Crédit Agricole Franche-Comté, Région Bourgogne Franche-Comté, and the office of Mme Annie Genevard of the Assemblée Nationale. The high-throughput sequencing results were partly produced through the genotyping and sequencing facilities of ISEM (Institut des Sciences de l'Evolution-Montpellier) and Labex Centre Méditerranéen Environnement



Biodiversité. Analyses were performed on the Centre de Biologie pour la Gestion des Populations HPC computational platform thanks to Alexandre Dehne-Garcia, and on the supercomputer facilities of the Mésocentre de calcul de Franche-Comté. This work was carried out within the framework of the Zone Atelier Arc Jurassien (<http://zaaj.univ-fcomte.fr>.)

Thank-you to everyone who participated in journal club, especially Benjamin Dietre (who did double duty as my Latex-guru) and Javier Fernandez-de-Simon, and to Ahmed Abd-Elmola. Thanks (and hugs) to Riya Sharma, Diane Gold, Julia Madison and Jared Daley for their encouragement from across the sea.

Lastly I'd like to thank my family; a nine hour time difference hasn't been easy but the care packages, conference calls, and love have made it easier. Thanks to my mum and dad for believing in me, and thanks to my sister Lexa for being a constant source of inspiration and a soundboard like no other, and for helping me trap voles while on your vacation (again); I promise, that was the last time...



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# Publications Associated with the Thesis

## Publications

P. Villette, E. Afonso, G. Couval, A. Levret, M. Galan, C. Tatard, J.F. Cosson, and P. Giraudoux (2017). Consequences of organ choice in describing bacterial pathogen assemblages in a rodent population. *Epidemiology and Infection*, 145 (14):3070-3075. doi:10.1017/S0950268817001893

## Poster Presentations

P. Villette, E. Afonso, G. Couval, A. Levret, M. Galan, C. Tatard, J.F. Cosson, and P. Giraudoux (2016). Metabarcoding of Bacterial Pathogens in a Rodent Pest: Which Organ? *Society for Molecular Biology and Evolution Conference (SMBE 2016)*, July 3-7 2016, Gold Coast, Australia

P. Villette, E. Afonso, G. Couval, A. Levret, M. Galan, C. Tatard, J.F. Cosson, and P. Giraudoux (2016). Rolling the dice: Looking for bacteria in water voles. *Forum des Jeune Chercheurs 2016*, June 16-17 2016, Université de Bourgogne Franche-Comté UFR SMP.



# Introduction

The relationship between people and rodents (Order Rodentia) is an old one; early accounts show clearly that rodents were a destructive agent in the fields and a source of disease for many ancient societies, and the mystery surrounding their devastation often led to them being seen as manifestations of divine will. For example, mouse "plagues" among the Philistines are attributed to God's wrath in the the Old Testament (Brenton, 1844), and among the ancient Greeks Apollo Smintheus ("mouse Apollo") was the god of field mice as agents of famine and of disease (Powell, 1929).

A recurring pattern in these accounts is the somewhat ephemeral nature of rodent destruction; as Charles Elton (1942) observed, many of these stories follow the same plot:

"Voles multiply. Destruction reigns. There is dismay, followed by outcry, and demands to Authority. Authority remembers its experts or appoints some: they ought to know. The experts advise a Cure. The Cure can be almost anything: golden mice, holy water from Mecca, a Government Commission, a culture of bacteria, poison, prayers denunciatory or tactful, a new god, a trap, a Pied Piper. The Cures have only one thing in common: with a little patience *they always work*. They have never been known entirely to fail. Likewise they have never been known to prevent the next outbreak. For the cycle of abundance and scarcity has a rhythm of its own, and the Cures are applied just when the plague of voles is going to abate through its own loss of momentum."

## 1.1 Rodent Population Fluctuations

These cycles of abundance were first noted by Elton when reviewing disease in wildlife (Elton, 1931); he observed that regular outbreaks of voles in the United Kingdom and Bavaria were well documented, but examples of irregular outbreaks in other regions and species also existed. Elton suggested that the apparent irregularity of these "violent fluctuations" in vole abundance observed in places like the French regions of Alsace and Aisne, and the fluctuations in lemming abundance in Norway and Canada, were due to variations in the magnitude of the outbreak; i.e. that regular cycles existed in these

populations, but some peak-year abundances were sufficiently low to escape casual human notice.

Since then, the study of rodent population outbreaks has generated a large body of literature (Batzli, 1992) and a better understanding of the variety of patterns of population dynamics that Elton first alluded to. Figures 1.1 and 1.2 show some of this variation graphically. Figure 1.1.A is a time series of population density of a red-backed vole (*Myodes rutilus*) population that has been under study since the early 1970s (Boonstra et al., 2001) and it cycles with a period of 3-5 years (Boonstra and Krebs, 2012). Figure 1.1.B is a population of *Lemmus lemmus* in Sweden that cycles with a periodicity of 3.4 years (Framstad et al., 1997). These first two examples exhibit the classic 3-5 year cycle that Elton first described. Figure 1.1.C and 1.1.D are less clearly cyclic; a *Microtus ochrogaster* population in Illinois cycles with a period of 4.3 years (Fig. 1.1.C, Getz et al., 2001), but much of the multiannual variation is obscured by the sampling frequency (every two weeks). The *Arvicola terrestris* series from eastern France (Figure 1.1.D) is one of 314 time series collected over 2500 km<sup>2</sup> that when analyzed together have a mean period of 6 years (Berthier et al., 2014).

But not all rodent populations are cyclic; indeed, within a single species one may find populations with relatively stable dynamics, or infrequent outbreaks, or regular outbreaks. Figure 1.2 (modified from Delattre et al., 1992) is a set of abundance time series of 6 populations of *Microtus arvalis* in France that illustrates this point. If we classify each population based on the amplitude of fluctuations, the temporal scale of the amplitudes (seasonal vs. multiannual), and whether populations experienced periodic local extinctions, as the authors did, then we find 4 different types of population dynamics in six populations (see figure caption for details).

Krebs (2013) has described two paradigms that can be adopted to approach this variation in rodent population dynamics: the first identifies each case as unique and seeks to identify a unique explanation for each case; the second views all cases as belonging to a set of related phenomena, and seeks to identify a common mechanism responsible for generating all of the patterns observed. If adopting the latter approach, as we intend to do here, then questions regarding each population can be unified under one underlying question: why don't populations increase indefinitely?

In order to approach this question, we must first define a common terminology and identify similarities in rodent fluctuations across populations and species. Figure 1.3 illustrates the shape of an outbreak as described by Krebs and Myers (1974), in which a single outbreak can be broken up into phases defined by density and population growth rate. The increase phase is characterized by a population growth rate greater than 1, leading to an increase in population density. The peak phase is characterized by a low population growth rate but high density. The decline phase features a population growth

rate less than 1 and consequently a decrease in population density, and the low or trough phase is characterized by low population growth rate and low density.

Figure 1.3 also summarizes some of the demographic characteristics that appear in some of the populations that exhibit outbreaks. High juvenile recruitment coupled with early female maturation and a longer breeding season appear to be the drivers behind increasing population densities in at least some of the rodent populations that undergo violent population fluctuations, and lower juvenile survival in conjunction with delayed maturation and a shorter breeding season appear to be conserved demographic characteristics of the decline phase of many of these outbreaks. Adult survival may fluctuate with cycle phase (e.g. Chitty, 1952) but this does not appear to be universal (e.g. Boonstra, 1985), and isn't expected to be a significant variable effecting population growth rate (Oli and Dobson, 1999). The proportion of reproductive females also does not appear to change with cycle phase in cyclic populations (Norrdahl and Korpimäki, 2002).

## 1.2 Hypotheses

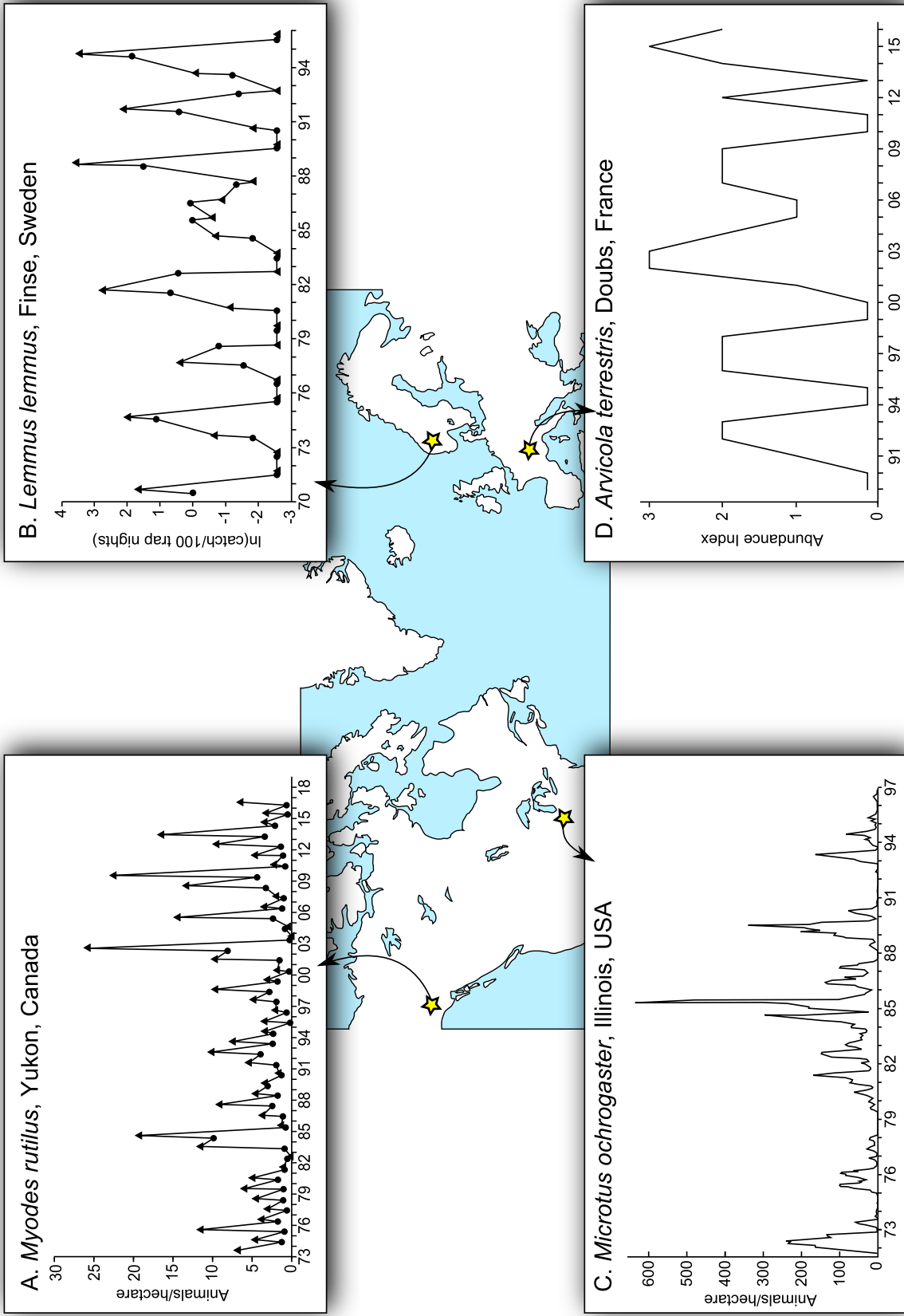
Hypotheses regarding rodent outbreaks have traditionally been organized into several themes: the food hypothesis, predation hypothesis, self regulation hypothesis, and the disease hypothesis (Krebs, 2013). How these hypotheses have been tested has typically followed one of two approaches: a single-factor deductive approach advanced by Dennis Chitty, in which factors are tested with experiments *a la* Popper's falsification approach to science, or a multi-factorial inductive route advanced by Bill Lidicker, which relies on the accumulation of evidence to support hypotheses that are multi-factorial in nature (Krebs, 2013). What unifies these approaches is the emphasis on delayed density dependence, or that density-dependent regulation is not instantaneous but operates with a time lag, because this is expected to generate cyclic outbreaks like those seen in Figures 1.1 and 1.2 (Berryman and Kindlmann, 2008; May, 1973).

### **Box 1.2: Hypotheses**

Research regarding rodent population dynamics, and multiannual fluctuations particularly, has been ongoing for over 100 years and has generated a considerable body of scientific literature (Batzli, 1992). We refer readers to Krebs (2013) for an in-depth overview of that literature and the various hypotheses and mechanisms that have been proposed over the years; here, we present a brief summary of the main categories into which many of these hypotheses can be grouped: food, predation, and self-regulation. Discussion of the disease hypothesis appears in the main text.

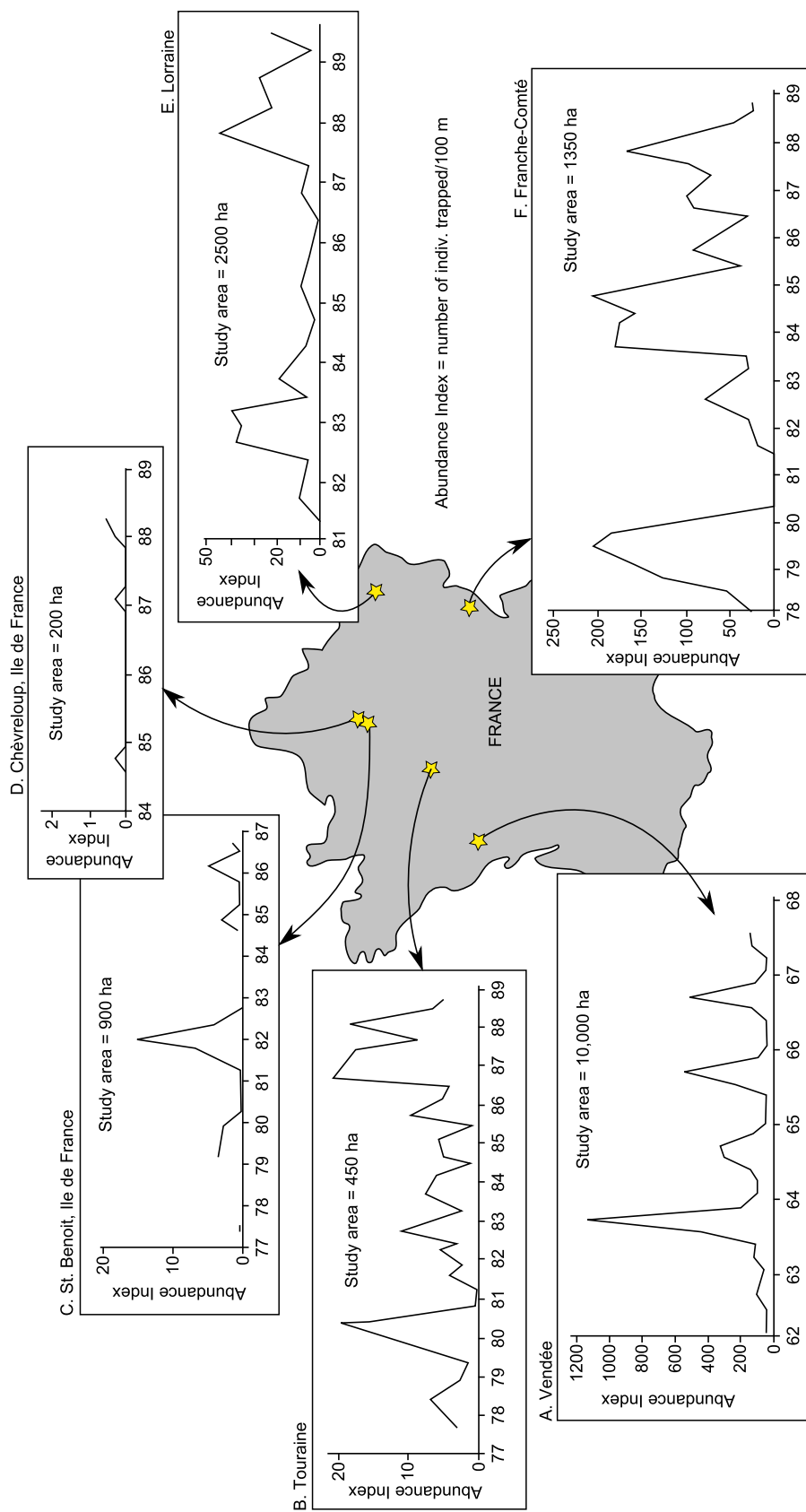
**Food limitation** - Food limitation was one of the early hypothesis put forward to explain the catastrophic declines observed following outbreaks, and have ranged from simply stating





**Fig. 1.1.:** Four examples of rodent population dynamics from the Northern Hemisphere.

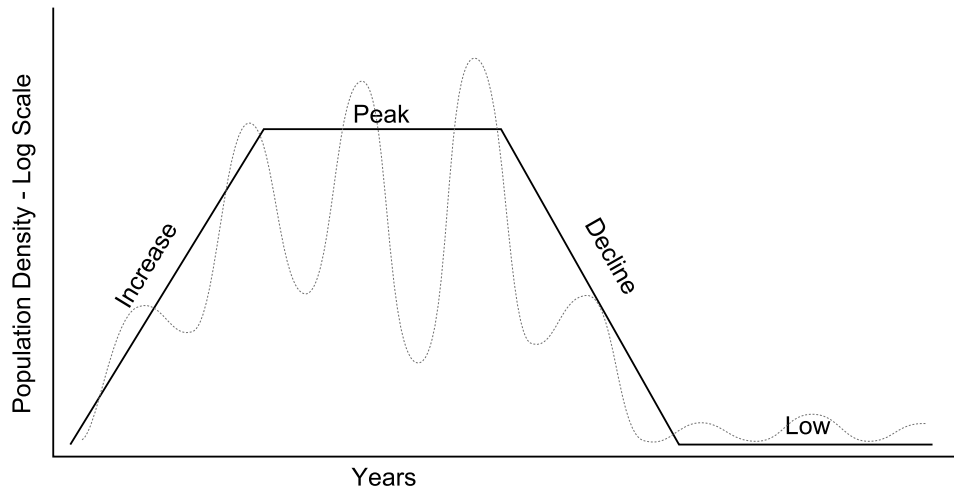
- A) The red-backed vole, *Myodes rutilus*, at Kluane Lake, Yukon Territory, Canada. Data are average densities from three 2.25 ha grids located in boreal forest, estimated using spatially-explicit capture recapture models applied to live-trapping data collected from 2-3 days of live-trapping in May (spring, circles) and August (autumn, triangles) every year from August 1974 to August 2017. Data courtesy of Charles Krebs.
- B) The Norwegian lemming, *Lemmus lemmus*, at Finse, Sweden. Data are abundance indices (natural logarithm of number of animals caught per 100 trapping nights) from one 1 ha alpine grid snap trapped in late June/early July (spring, circles) and late August/early September (autumn, triangles) every year from 1970 to 1996. Data from Framstad et al. (1997).
- C) The prairie vole, *Microtus ochrogaster*, in an alfalfa field outside of Urbana, Illinois, USA. Data are densities from 5 adjacent 1-1.4 grids (grid Alf I from May 1972 - March 1977; grid Alf II from March 1977 to July 1984; grid Alf III from October 1983 to December 1989; grid Alf IV from January 1990 to June 1993, and grid Alf V from July 1993 to May 1997). Grids were live-trapped monthly, and densities were estimated as minimum number known to be alive (MNA) divided by grid size. Data from Getz et al. (2001)
- D) The fossorial water vole, *Arvicola terrestris*, in the commune of Arc-sous-Montenot, Doubs department, Franche-Comté, France. Data are qualitative abundance indices assessed at the commune level (25 km<sup>2</sup>) every autumn by vehicle; 0 - no water vole colonies (indicated by fresh tumuli) observed in the commune, 1 - isolated active colonies in some parcels in the commune, 2 widespread colonization in many of the parcels in the commune, not all colonies are connected, 3 - all parcels in the commune feature active and well-connected colonies. Data courtesy of Geoffroy Couval of the FREDON-FC (Fédération Régional de Défense contrôles Organismes Nuisibles de Franche-Comté)



**Fig. 1.2.:** Six populations of *Microtus arvalis* sampled several times a year in France. Study sites were sampled using trap lines; the abundance index is the number of animals trapped/100 m. Note that the vertical axis differs between series. Delattre et al. (1992) classified each population based on whether changes in abundance were multiannual or seasonal in duration, whether amplitudes were high or low, and whether the population experienced periodic local extinction:

- A. **Vendée** exhibits Type V oscillations (seasonal high-amplitude oscillations with no periodic local extinctions).
- B. **Touraine** exhibits Type III fluctuations (multiannual low-amplitude fluctuations without periodic local extinctions).
- C. **St. Benoit, Ile de France** exhibits Type IV fluctuations (multiannual high-amplitude fluctuations with periodic local extinctions).
- D. **Chèvreloup** exhibits Type I fluctuations (multiannual low-amplitude fluctuations with periodic local extinctions).
- E. **Lorraine** exhibits Type IV fluctuations (multiannual high-amplitude fluctuations with periodic local extinctions).
- F. **Franche-Comté** exhibits Type IV fluctuations (multiannual high-amplitude fluctuations with periodic local extinctions).

Figure adapted from (Delattre et al., 1992)



Pattern	Increase	Peak	Decline	Low	Examples
Female Maturation	Early		Late		<i>Myodes rufocanus</i> , Finland (Kalela 1957 in Krebs 2013) <i>Myodes rutilus</i> , Soviet Union (Koshkina 1965 in Krebs 2013) <i>Myodes glareolus</i> , Poland (Bujalska, 1985) lemmings, Canada (Krebs, 1964) <i>Microtus sp.</i> , North America (Keller and Krebs, 1970)
Breeding Season	Long		Short		<i>Myodes rufocanus</i> , Finland (Kalela 1957 in Krebs 2013) lemmings, Canada (Krebs, 1964)
Nestling Survival	High		Low		<i>Microtus californicus</i> , USA (Krebs, 1966) <i>Microtus ochrogaster</i> , USA (Getz et al., 2000)
Juvenile Survival	High		Low		<i>Microtus californicus</i> , Canada (Boonstra, 1985) <i>Microtus ochrogaster</i> , USA (Getz et al., 2000)

**Fig. 1.3.:** Phases of an outbreak, modified after Krebs and Myers (1974). Note that abundance is log-transformed; a hypothetical untransformed density time series is indicated with a dashed line showing seasonal fluctuations (see also Figure 1.1.A and 1.1.C). Demographic changes that have been observed over the four different phases of the cycle are listed below, with example populations.

that population growth is related to the availability of food, to the specification of a plant or nutrient that limits population growth (Lauckhart, 1957; Braestrup, 1940). An alternative formulation of the food limitation hypothesis is the food-habitat quality hypothesis, which makes predictions regarding the carrying capacity of a landscape as opposed to population growth rates; higher-quality habitat supports more food and therefore can support more animals (Frank, 1957). Food hypotheses as singular explanations of the outbreak and collapse have been largely rejected, due to observations regarding food abundance (e.g. *Microtus agrestis* in the U.K., Summerhayes, 1941) or food quality (nutrients or toxin levels, Bergeron and Jodoin, 1993), and food-addition experiments have failed to prevent population collapse from occurring (e.g. Cole and Batzli, 1978; Taitt et al., 1981).

**Predation** - Predation has also been suggested as a driver of rodent population fluctuations; predator-prey theory predicts that specialist predators, or those that feed on one or a few kinds of prey, can destabilize prey populations because they exert delayed- and direct density dependent mortality on their prey populations, while generalist predators, that feed on a wide variety of prey species, inflict direct density dependent mortality and therefore will stabilize prey populations. Tests of this prediction, in the form of predator removal experiments, have provided evidence both supporting and rejecting this hypothesis. Korpimäki and Norrdahl (1998) removed all predators during the summer following a decline, which led to significant increases in *Microtus* density compared to controls and treatment areas where just specialist predators (i.e. weasels) were removed. Follow up predator removal studies found that removing all predators also increased vole densities in increase and peak phases (the effect of just removing mustelids was not studied) (Korpimäki et al., 2002). In contrast, in Scotland, least weasel removal failed to alter vole densities and increased juvenile survival rates (Graham and Lambin, 2002).

**Self Regulation** - Dennis Chitty proposed that populations could self-regulate, and that a deterioration in individual quality as population density increased prevented population density from increasing indefinitely (Chitty, 1960). He hypothesized that self-regulation could be achieved through interactions centered around spacing behaviour leading to genetic changes in birth, death and dispersal rates that could then be subject to natural selection (Chitty, 1967). Lab and field experiments have found little evidence of the high levels of heritability in life history traits necessary for the genetic formulation of this hypothesis to be supported (Anderson, 1975; Boonstra and Boag, 1987), and as such it has been largely rejected.

An alternative formulation of the self-regulation hypothesis is that physiological stress due to social interactions at high density leads to increased mortality and reduced birth rates, instigating the decline (Krebs, 2013). Early tests of the stress version of the self-regulation hypothesis relied on crude measures of stress that are now obsolete, and also stipulated that social interactions be the source of stress; under these terms, the hypothesis was largely rejected when little evidence of physiological stress (measured using adrenal gland size) correlating to cycle phase was found in wild populations (Chitty, 1961). More recent investigations into this hypothesis have adopted better measures of physiological stress, and have stipulated that stress can be due to factors other than social interactions (e.g. predation or food shortages Sheriff et al., 2009; Charbonnel et al., 2008b).

The disease hypothesis was the original explanation proposed by Elton when he summarized rodent population outbreaks (see Box 1.2 for overviews of the other hypotheses); he argued that disease was the cause of the decline (Elton, 1931), and as proof, he pointed to Horne's isolation of *Bacillus pestis-lemmi* (*Yersinia pestis*) in four outbreaks of Norwegian lemmings (Horne, 1912) and Collett's observation of high prevalences of a *Streptothrix* (*Streptobacillus*) *lemmani* - associated skin infection in high-density lemming years (Collett, 1895). It appeared evident to him that "epidemic disease" was the cause of the decline. But when he and his colleagues attempted to identify the cause of death during a period of poor health and high mortality in a cyclic population of *Apodemus*, no evidence of a bacterial or viral epidemic was found (Elton et al., 1931).

Interest in pathogens and parasites as a driver of cyclic declines was maintained with the discovery of *Toxoplasma* in the brains of Welsh *Microtus* during a decline (Findlay and Middleton, 1934); but the subsequent failure to find significant levels of *Toxoplasma* infection in later declines undermined the hypothesis that *Toxoplasma* alone could explain cyclic declines (Elton, 1942). *Mycobacterium tuberculosis*, the causative agent of tuberculosis, was also hypothesized to cause declines after its discovery in *Microtus agrestis* across the British Isles (Wells and Oxon, 1937), but was eventually discarded when Chitty (1954) reported no correlation between tuberculosis prevalence and density in some *Microtus* populations, and observed very low tuberculosis prevalences in some declining populations. Chitty's conclusion that pathogens and parasites were unlikely to drive population declines in any species, and his development of the self-regulation hypothesis as an alternative explanation for the cycles, led to the widespread acceptance that diseases were not important in the cycles; this view would go unchallenged in the rodent community for over 40 years.

But research into the regulatory potential of parasites in wildlife populations continued outside of the rodent cycle sphere. Anderson and May's seminal work modelling infectious diseases in animal populations (Anderson and May, 1978; May and Anderson, 1978; Anderson and May, 1979; May and Anderson, 1979), in which host population abundance was permitted to vary within the model and not be held constant, provided a mathematical framework with which to explore host-parasite dynamics, and also identified characteristics of a host-parasite system that may generate oscillations in host abundance: parasite-induced reductions in host fecundity, direct reproduction of the parasite within the host increasing parasite population size, and delays in parasite reproduction and transmission (May and Anderson, 1978).

These models were applied to the red grouse (*Lagopus lagopus scoticus*) - nematode (*Trichostrongylus tenuis*) host-parasite system in England, in which some red grouse populations fluctuated multiannually with a mean period of 4-8 years. The dominant hypothesis in the 1970s to explain these cycles was spacing behaviour (Hudson et al., 2002), but Dobson and Hudson (1992) used the Anderson-May models to develop

an alternative hypothesis regarding the nematode as the factor driving the cycles via reduced fecundity in infected females. Experimental manipulations of parasite burden supported the model predictions; treating 20 % of the grouse population with an anti-helminth reduced the amplitude of the multi-annual fluctuations (Hudson et al., 1998).

Since then, there has been renewed interest in parasites as a potential important factor in rodent cycles, with an ever widening suite of infectious agents coming under scrutiny. For example, Soveri et al. (2000) found a range of pathogens and histopathological changes to be common in declining populations of *Myodes glareolus* and *Microtus agrestis*, notably widespread infection by *Taenia mustelae* and *Bordetella bronchiseptica*, increases in lymphoid tissue in the lungs, and enteritis. Cavanagh et al. (2004) reported that antibodies to cowpox virus and clinical signs of *Mycobacterium microti* infection in cyclic populations of *Microtus agrestis* in England exhibited delayed density dependence with a lag of three to six months. Niklasson et al. (1995) detected delayed density dependence in Puumala virus antibodies in a cyclic *Myodes glareolus* population in Sweden. Charbonnel et al. (2008a) explored the prevalence of antibodies to Puumala virus, lymphocytic choriomeningitis virus, and cowpox virus in cyclic populations of *A. terrestris* in Franche-Comté and reported that cowpox antibody prevalence was highest in populations that were at high abundance the current and previous autumn (what one might call the late peak phase). These studies have looked for correlations between disease prevalence and cycle phase, but there have been some experimental manipulations as well: Pedersen and Greives (2008) found that only a combination of both food addition and anti-helminth treatments prevented population declines in mixed *Peromyscus leucopus* - *Peromyscus maniculatus* populations that cycle in response to oak acorn masts.

When not manipulating helminths, the focus has been on patterns of prevalence of parasite species, often considered in isolation - Charbonnel et al. (2008a) briefly comment on co-infections of cowpox and lymphocytic choriomeningitis virus but samples sizes were too low to explore this phenomenon further, and Soveri et al. (2000) didn't report on coinfections at all. But coinfections of micro- and macroparasites have been reported in many hosts and are important because they can influence host susceptibility to other infections (Cox, 2001), the course of the disease (Chertow and Memoli, 2013) as well as the transmission dynamics of the parasites involved (Abu-Raddad et al., 2006).

A particularly clear example of how parasite interactions may shape host susceptibility can be found in cyclic *Microtus agrestis* populations in Kielder, England; using a longitudinal data set, Telfer et al. (2010) reported that infection with the bacterium *Anaplasma phagocytophilum* significantly increased the risk of infection by cowpox virus and the protozoan *Babesia microti*, and infection with cowpox virus could increase risk of infection by *A. phagocytophilum*, *B. microti*, and *Bartonella* sp. Thus, the patterns



observed by Cavanagh et al. (2004) regarding cowpox prevalence in Kielder voles may be related not only to abundance, as they found, but also other parasites in the system that they did not observe.

## 1.3 Parasites

We've discussed how individual parasites might influence host population dynamics and the dynamics of other parasites in the system, but relatively little attention has been paid to how the parasite community as a whole may affect and be affected by cyclic host population dynamics. A notable exception is the work by Cerqueira et al. (2007), in which they measured helminth parasite richness in cyclic populations of *A. terrestris* in Franche-Comté and found that species richness within individual hosts was highest in late-peak and declining populations. Whether increased helminth richness contributed to the decline or was a consequence of other factors was not elucidated; the authors didn't believe that a single helminth species could cause the decline, but synergistic effects were not dismissed either.

The result that host helminth richness changed over the course of the cycle intersects with some of the work conducted in parasite ecology in recent years that has focused on identifying common patterns in parasite community richness and structure within and between host species (see Poulin, 2014, for an overview). Much of this work has focused on macroparasites like helminths and ectoparasites, and hasn't explicitly addressed how parasite communities might change when host populations exhibit density or abundance fluctuations on the order observed in cyclic rodent populations, but we can still use these findings to inform our predictions about parasite communities in host populations that experience outbreaks (see Box 1.3 for terminology definitions).

### **Box 1.3: Parasite Ecology Terminology**

We use the terminology presented in Poulin (1995) in the following discussion and rest of the thesis:

**Infracommunity** - all individuals of all parasite species infecting a single individual host

**Component Community** - all individuals of all parasite species found in the host population

Component community richness is expected to be higher at high population density for a number of reasons. One involves the basic reproductive number  $R_0$  of a parasite species, which is a measure of parasite invasiveness. For microparasites, this is the number of secondary infections produced by the first individual parasite to enter a population of fully susceptible hosts, and is calculated as a ratio of host population density and parasite

transmission efficiency to natural and parasite-induced host mortality (Anderson and May, 1991; Dieckmann and Heesterbeek, 2000). When  $R_0$  is less than 1, the parasite cannot maintain itself and declines to local extinction - thus at higher population densities, the  $R_0$  for more species will be above 1 and more species will persist in the host. Larger populations are also expected to host more species because they function as larger "targets" in much the same way as islands do in island biogeography theory; more individuals increases the likelihood of parasite transmission from another host species (Poulin and Morand, 2004). These patterns are predicted for host populations at equilibrium; one question we might ask is whether we see similar patterns in fluctuating populations (i.e. at low density, the population hosts fewer parasite species, and at higher density, the population hosts more parasite species).

Infracommunity richness is thought to be determined by the rate at which a host can be colonized by parasites and the duration or persistence of the subsequent infection (Poulin, 1995). Intrinsic host characteristics like immunocompetence may influence the rate of colonization by preventing the establishment of an infection, and may influence the duration or intensity of the infection (see Beldomenico et al. (2008a) for a review of how host condition and infection interact). Age may also play a role if infections are life-long; older individuals will have had more time to accumulate parasites and may therefore be expected to host richer infracommunities (e.g. *Echinococcus multilocularis* in *A. terrestris* and *Microtus arvalis*, Burette et al., 2011; Delattre et al., 1988). Extrinsic factors like population density are expected to influence transmission rates for some, but not all parasites, and seasonal changes in the environment and the associated metabolic demands may also play a role (Beldomenico et al., 2008b). The parasite pool hosted by the population also limits infracommunity richness, thus component community richness and infracommunity richness may also be related (Poulin and Morand, 2004). Cerqueira et al. (2007) observed delayed density dependence in infracommunity helminth richness, but it is unclear if we should expect to see this pattern in directly transmitted parasites like some bacteria or viruses.

Thus, we have the following observations: models and some empirical evidence support the idea that parasites can generate population cycles in hosts; delayed density dependence of parasite species prevalences has been observed in some cyclic populations, and this correlation can be interpreted as weak evidence that parasites play a role in the population dynamics of these hosts; parasites are likely to interact with each other within host populations, which advocates a multi-parasite approach when studying parasites in host populations; and parasite communities are expected to vary with characteristics of the host population but synergistic effects of parasite communities on host populations are unclear. In light of these observations, we are interested in exploring parasite communities in a cyclic rodent populations. We use the fossorial water vole, *A. terrestris*, described in the following section, as a model system to explore bacterial parasite communities in rodent hosts.

## 1.4 *Arvicola terrestris*

The focus of this thesis is on the fossorial form of the water vole, *Arvicola terrestris* in the Doubs and Jura departments of Franche-Comté, eastern France (see Box 1.1 for a discussion of the taxonomy of this genus). In this region, *A. terrestris* are found primarily in grasslands above 400 m above sea level (Giraudoux et al., 1997). Adults range in mass from 80-160 grams, and measure between 12 and 18 cm from head to anus (Delattre and Giraudoux, 2009). Females reach sexual maturity within 5 weeks and are capable of having up to 3 litters per year with an average of 4-5 embryos per litter (Delattre and Giraudoux, 2009). They inhabit permanent grassland, pasture, and cultivated parcels, digging galleries approx. 20 cm under the surface (range: 5 cm to 56 cm) and an average of 40.2 m in length; gallery size and complexity varies with population density, altitude, season, and the age of the galleries (Airoidi, 1976b). Galleries are occupied by a mix of males and females, adults and juveniles (Airoidi, 1976a) and above-ground dispersal tends to occur among juveniles and sub-adults of both sexes during periods of rainfall (Saucy and Schneider, 1997).

Within a socio-economic context, *A. terrestris* is a significant species in this region because not only does it cause significant agricultural losses during periods of high abundance (detailed below) but it also hosts a variety of organisms that pose significant health risks to humans. In particular, it is an intermediate host for the helminth *Echinococcus multilocularis*, a parasite that targets the liver and grows much like a tumour; untreated, this parasite can be lethal in humans (Eckert and Deplazes, 2004). *A. terrestris* can also host *Francisella tularensis*, a virulent intracellular bacterium that can, depending on the strain, cause severe disease (Oyston, 2008). Risk of infection by both of these organisms in humans increases with increasing water vole abundance (Viel et al., 1999; Efimov et al., 2003). Rodent species that reach sexual maturity quickly and can produce large litters early in life are more likely to be significant reservoirs for zoonoses (Han et al., 2015) making *A. terrestris*, with its capacity for explosive population growth, of particular interest as a potential reservoir for emerging infectious diseases.

### **Box 1.1: *Arvicola* taxonomy**

Since the 1970s, researchers have recognized between two and three species of *Arvicola* sp.; *A. sapidus*, an aquatic form found in the Iberian peninsula and France, *A. amphibius*, an aquatic form found from Western Europe to Eastern Siberia, and *A. scherman*, a fossorial form found in the mountainous regions of northern Spain, the Pyrenees, the Alps, the Carpathians, and the mountains of Central Europe. *A. amphibius* and *A. scherman* are sometimes listed as subspecies of *A. terrestris* (e.g Duhamel et al., 2000; Stoddart et al., 1975). *A. sapidus* is well defined taxonomically due to the number of chromosomes it possesses ( $2n=40$ , as opposed to  $2n=36$  for *A. scherman* and *A. amphibius*, Guardia and



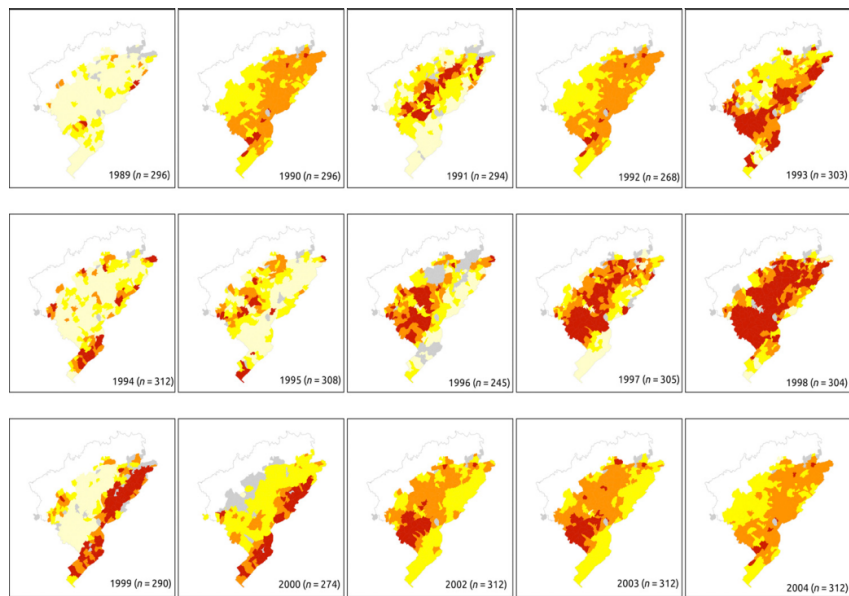
**Fig. 1.4.:** Adult *Arvicola terrestris*. Photo credit: Petra Villette

Pretel 1979), but recent molecular work has failed to find genetic support for *A. amphibius* and *A. scherman* as reciprocally monophyletic species delineated by ecotype. Kryštufek et al. (2015) identified one monophyletic clade composed of both fossorial and aquatic types, distributed from central Europe to eastern Siberia, and a much smaller monophyletic clade composed strictly of fossorial voles from eastern Switzerland. As of this writing, naming conventions for these clades have not been established, and the clade to which Franche-Comté fossorial water voles belongs to is unknown. As such, we refer to the fossorial water voles of Franche-Comté as *A. terrestris*.

Population densities of *A. terrestris* in Franche-Comté fluctuate dramatically, with outbreaks characterized by densities of over 200 individuals/ha and forage production losses of over 1000 kg/ha/year occurring every 5-8 years (mean period, 6 years, Berthier et al., 2014). Fluctuations in *A. terrestris* density have been documented in neighbouring Switzerland as early as the 16th century (Korner, 1993; Saucy, 1994b), but current outbreaks of such magnitude emerged in the late 1960s in Franche-Comté in conjunction with significant land-use changes; agricultural policy at the time encouraged specialization in milk production which led farmers to convert ploughed fields to permanent grassland (Giraudoux et al., 1997) and remove hedges to create larger homogeneous patches.

Some of the demographic patterns listed in Figure 1.3 also apply to water vole outbreaks; declining populations tend to be older than peak populations and this has been attributed to poor juvenile survival (Cerqueira et al., 2006). To our knowledge, timing of female sexual maturity (age at first litter) in relation to cycle phase has not been examined in this system, nor has the length of the breeding season, and further investigations into these questions are warranted.

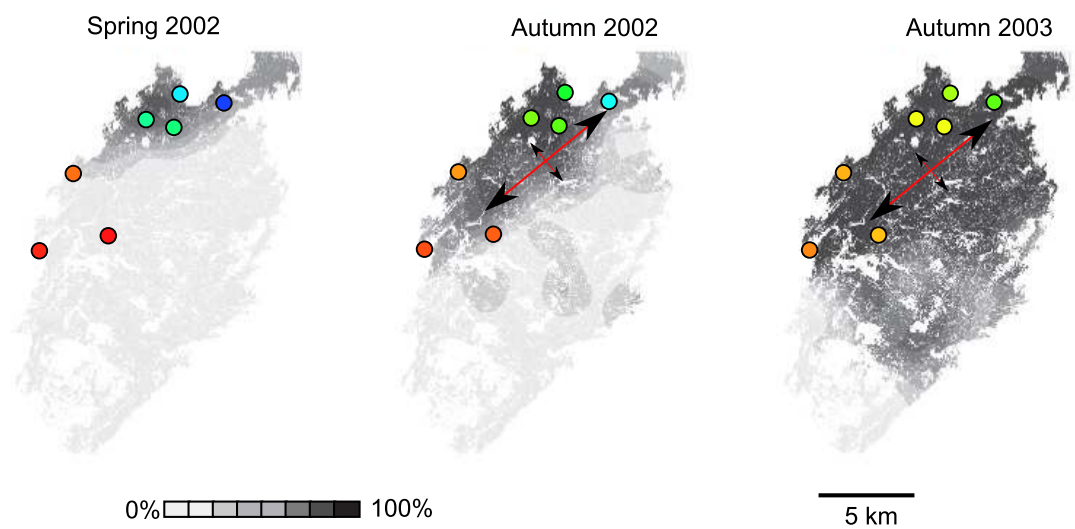
Risk of outbreak is predominantly mediated by the availability of permanent grassland (which is not plowed or harrowed, nor intensively grazed); this allows for the persistence of mole *Talpa europaea* galleries which in turn facilitates colonization of the grassland by *A. terrestris* (Delattre et al., 2006). *A. terrestris* and *T. europaea* will use the same



**Fig. 1.5.:** Water vole abundances at the commune scale for each year from 1989-2004 in the Doubs department. *n* is the number of communes from which data was recorded. Colour indicates voles abundance: red is high abundance, orange is moderate abundance, yellow is low abundance, pale yellow is absence and grey is no data. From Berthier et al. (2014)

tunnel networks when *A. terrestris* density is low (Giraudoux et al., 1995; Fritschy and Meylan, 1980). At the regional scale ( $\sim 2500 \text{ km}^2$ ), risk of outbreak is highest when the ratio of permanent grassland to agricultural land is  $>90 \%$  but outbreaks still occur at ratios as low as 40% (Giraudoux et al., 1997). At the commune scale ( $\sim 25 \text{ km}^2$ ), outbreaks tend to occur in areas of homogeneous grassland as opposed to areas broken up with hedge networks and forest patches (Duhamel et al., 2000). At a local scale of  $\sim 2.5 \text{ km}^2$ , outbreaks reach high abundances in larger-area grassland patches, and at  $\sim 0.5 \text{ km}^2$ , outbreaks peak earlier in patches with greater relative grassland area and lower grassland-forest edge densities (Morilhat et al., 2008).

*A. terrestris* outbreaks in Franche-Comté, like other examples of rodent cycles, exhibit synchrony over a large geographic area, but *A. terrestris* outbreaks exhibit lagged synchrony, leading to a travelling wave that sweeps across the landscape at approx. 7.4 km/year (Berthier et al. 2014; Figure 1.5). The epicentre of the outbreak is focused on the geographic transition between two plateaus (400-700 m and 700-900 m in elevation) which runs in a north-east/south-west direction; the travelling wave sweeps south-east, almost perfectly perpendicular to direction of the geographic transition (Berthier et al., 2014). Gene flow is higher along the wave front than in the travel direction of the wave (Berthier et al., 2014), which is likely related to the observation that rates of successful immigration are higher between adjacent populations that are both at high abundance as opposed to adjacent populations that are out of sync (Berthier et al., 2006).



**Fig. 1.6.:** *Arvicola terrestris* abundance in the grasslands of Nozeroy canton, Franche-Comté in Spring 2002 and Autumn 2002 and 2003. Grey shading indicates local abundance of water voles, circles are sampling sites in which individuals were collected for genetic analysis. Genetic similarity between sampling sites (measured as global  $F_{st}$  and pairwise  $F_{st}$  comparisons) was lower in spring 2002 (represented by different-coloured circles) and increased as abundances increased (colours become more similar). Arrows indicate dispersal direction during this period; the majority of dispersal occurred towards the north-east and south-west. Figure based on Figure 2.3 in Delattre and Giraudoux (2009) and data from Berthier et al. (2006).

*A. terrestris* outbreaks in this region are somewhat unique in that at the local (farm) scale, the high density phase can persist for between 1 and 3 years. Giraudoux et al. (1997) reported that the duration of the high-density phase is somewhat positively correlated with the ratio of permanent grassland to agricultural land and negatively correlated with the ratio of forest to agricultural land (Pearson's  $r = 0.29$  and  $-0.28$  to  $-0.16$  respectively, depending on densities reached during the peak of the outbreak), but a better understanding of why the peak phase can persist for some years during one outbreak but only one year during another outbreak, in the same location, is sorely lacking.

Early management responses to the outbreaks relied heavily on the application of pesticides, first at the local (farm) scale, then later coordinated at the commune scale (Delattre and Giraudoux, 2009). This approach did not effectively prevent outbreaks from happening, and also resulted in secondary poisoning of predators. Pressure from local hunting and environmental associations coupled with farmer's demands for effective management tools led to the development of a holistic "toolbox" of methods for reducing the risk of outbreak and limiting the damage when an outbreak does occur. This toolbox includes crop rotation and the associated plowing to destroy galleries, grazing of permanent grassland normally reserved for hay production (grazing serves to collapse tunnels), mole control to reduce parcel colonization by *A. terrestris*, and increasing edge habitat and habitat favourable for predators. Rodenticide use is strictly limited to when *A. terrestris* densities are low to reduce the risk of secondary poisoning (Couval and Truchetet, 2014; Coeurdassier et al., 2014).

Parasites and pathogens in *A. terrestris* populations in Franche-Comté has received some attention, especially in the context of human health; as discussed above, *A. terrestris* is the intermediate host for *Echinococcus multilocularis* and *A. terrestris* population dynamics play a role in the risk of infection in humans. Other helminths have also been studied: Cerqueira et al. (2007) reported the presence of 6 helminth species other than *E. multilocularis* in *A. terrestris* in Franche-Comté, and found that infracommunity richness was highest in late peak and declining populations.

Conversely, Deter et al. (2007) found that *Trichuris arvicolae* was most prevalent in increasing and peak *A. terrestris* populations and completely absent from declining populations. They suggested that this was due to differences in age structure between the different phases and dispersal; younger individuals were more frequently infected than older individual and more likely to disperse, and decline-phase populations had fewer juveniles than increasing and peak populations. Deter et al. (2008) developed a series of models for the effects of *T. arvicolae* on vole population dynamics and found that *T. arvicolae* is capable of regulating *A. terrestris* populations, reducing infected populations by as much as 50 %, but is unlikely to contribute to the population cycles.

*Taenia taeniaformis* is also found in *A. terrestris*, although infection doesn't appear to alter host body mass, litter sizes, or sexual activity (Deter et al., 2006). Prevalence increases with increasing host age, is higher in spring than in autumn, and is higher at low local (<0.1 km<sup>2</sup>) host abundance than during outbreaks; phase of the cycle and larger-scale (> 10 km<sup>2</sup>) *A. terrestris* abundance estimates were not strongly correlated with the prevalence of this parasite. Suggested explanations for this pattern included changes in spacing behaviour at low abundance increasing movement in the landscape and increasing exposure to *T. taeniaformis* ova, lags in predator response to increasing *A. terrestris* abundance during an outbreak resulting in fewer ova per *A. terrestris* in the environment available to infect new *A. terrestris* individuals, and poor condition during the decline phase increasing susceptibility to infection.

Viruses have also received some attention: Charbonnel et al. (2008a) detected antibodies to Puumala virus, lymphocytic choriomeningitis virus, and cowpox virus in *A. terrestris* and reported that cowpox prevalence was higher in late-peak populations than in early-peak and increasing populations. In addition, cowpox seroprevalence was spatially aggregated, with some study sites featuring high (> 70 %) seroprevalences while in others, cowpox antibodies were entirely absent.

## 1.5 Thesis Objectives

As in many other systems, little is currently known about the bacterial parasites hosted by *A. terrestris* in Franche-Comté. This paucity of information is in part due to technological constraints; prior to the development of relatively cheap high-throughput DNA sequencing technologies, identifying bacteria relied on several different methods, each with their own limitations. Labour- and time-intensive culture methods necessarily excluded species that are resistant to culturing, and culturing, like quantitative PCR, DNA arrays and antibody detection, all require some prior anticipation of the results, limiting the utility of these methods for exploratory analyses. But the development of approaches like 16S metagenomics, in which the 16S rRNA region of the bacterial genome, common to all bacteria, is sequenced, allows for the detection of bacteria without any *a priori* assumptions of what might be present in the sample of interest.

Screening for parasites in *A. terrestris* populations in Scotland revealed the presence of both unspecified *Bartonella* and *Leptospira* (Gelling et al., 2012), and *A. terrestris* has long been associated with *Francisella tularemia* in eastern Europe (Kudelina and Olsufiev, 1979). Bacterial epidemics in other species can be devastating (see Box 1.4 for notable examples), and it was data from a bacterial infection (*Muribacter* [*Actinobacillus*] *muris*) in a captive mouse population that was used as an example of parasite-mediated popula-



tion regulation in the influential papers by Roy Anderson and Robert May (Anderson and May, 1979; May and Anderson, 1979).

#### **Box 1.4: Examples of bacterial disease**

Bacteria are a major source of infection in humans and other animals. Particularly notable examples of bacterial disease outbreaks in humans include the 1347-1353 Black Death epidemic in Europe caused by *Yersinia pestis* (Haensch et al., 2010), estimated to have killed up to half of the population (Aberth, 2005), and the ongoing tuberculosis epidemic caused by *Mycobacterium tuberculosis*, which infects approximately 1.7 billion people worldwide and caused 1.6 million deaths in 2016 (World Health Organization, 2017).

Examples of wildlife populations impacted by bacterial infections include the population limitation of big-horn sheep in North America by *Mycoplasma*-associated bronchopneumonia (Besser et al., 2008), and *Chlamydia* infections in koalas reducing fecundity, which may have important implications for management and conservation of this species (Whittington, 2001).

Much of what is known about bacterial infections in rodents relates to the risks they pose to human health; Meerburg et al. (2009) extensively reviewed rodents as hosts of infectious agents that cause disease in humans, with notable examples included *Leptospirosis* in rodents worldwide, and *Borrelia* (the causative agent of Lyme disease) in mice, voles and hamsters in North America, Europe, and Asia. In many instances, the rodent host doesn't appear to be clinically affected by infection.

Thus, technological advances, coupled with the observed interactions between bacteria and other parasites observed in other cyclic rodent populations, the potential for bacterial parasites to effect host population dynamics, and interest from the local agricultural community in potential management applications of bacteria, have all contributed to the impetus behind this thesis, the general objective of which is to characterize the parasitic bacterial communities of *A. terrestris* populations in Franche-Comté during an outbreak and subsequent decline. Specifically, we focus on spatial and temporal variation in bacterial parasite infra- and component communities. *A. terrestris* populations in this region not only exhibit changes in abundance but also concomitant changes in population connectivity, as measured by gene flow; these changes in abundance and population isolation may be important in structuring local infra- and component communities both over the period of the outbreak but also over several outbreaks. We use animals collected in 2014-2016 from five communes in the eastern French region of Franche-Comté to address questions of spatial heterogeneity in parasite communities, and we use animals collected in 2003-2005 in three of the five communes to address questions of temporal heterogeneity in the regional parasite species pool.

In Chapter 2 of this thesis we describe the Franche-Comté region in which we conducted sampling, our sampling protocol and the sites we visited, as well as the molecular methods for extracting bacterial DNA from *A. terrestris* hosts, amplifying the V4 region of the 16S rRNA region of the bacterial genome, sequencing the resulting amplicons and filtering the resulting sequences for quality and contaminants. We also summarize the results of a pilot study we performed to answer the following question: which host organ should we use to look for parasitic bacteria? The spleen, because of its role in immune function and the clearing of bacterial infections, is a natural choice, but it is unclear if including additional organs would improve coverage of the host infracommunity. Using the hearts, livers, lungs, kidneys and spleens of 13 male *A. terrestris*, we assess how including additional organs increases the coverage of the host infracommunity.

In Chapter 3 of this thesis we describe the infra- and component communities of *A. terrestris* during the high abundance phase of the cycle. We focus on the high abundance phase because we believe that a firm understanding of the parasite communities found during the peak phase is necessary for forming and testing hypotheses regarding the parasite communities of the decline phase. We include animals from all five of our study sites, and we use bacterial DNA extracted from all five organs considered in our pilot study. We use generalized linear modelling to identify predictors of infra- and component community richness, to determine if bacterial parasite communities in this host vary spatially and temporally during the host high abundance phase. We also assess geographic distance as a correlate of infra- and component communities similarity through time.

In Chapter 4 we compare splenic bacterial communities of *A. terrestris* collected from three of our sites in 2003-2005 to splenic bacterial communities of *A. terrestris* that we collected in the same sites in 2015-2016. We perform a basic comparison of the bacterial OTUs detected in the two outbreaks to assess long-term heterogeneity in bacterial parasite communities in *A. terrestris*. We also use the IndVal approach (Dufrene and Legendre, 1997) to try and identify indicators of the late high-abundance phase of the outbreak, for use as a potential management tool for local agricultural professionals.

In Chapter 5 we relate the results of the previous three chapters to existing literature regarding parasites in cyclic rodent populations, discuss potential caveats of the approaches that we have taken, make predictions regarding future work planned for this system, and suggest further work necessary for understanding bacterial parasites in mammal hosts in general and *A. terrestris* populations specifically.



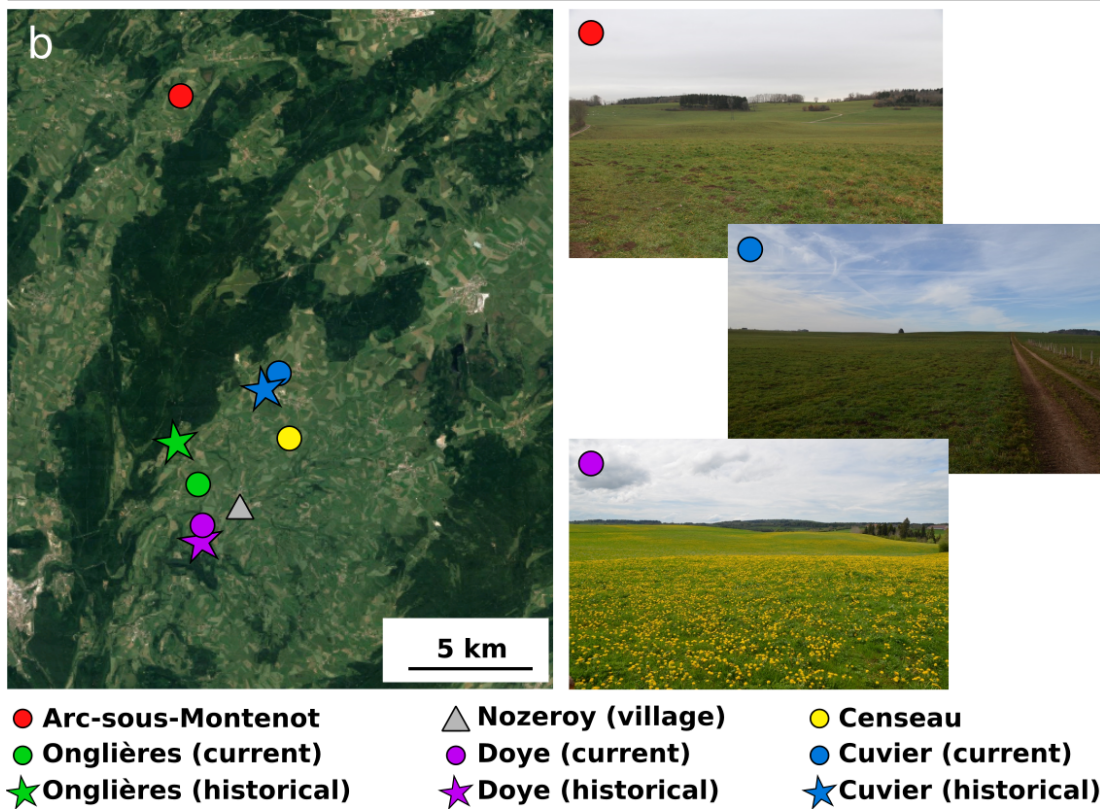
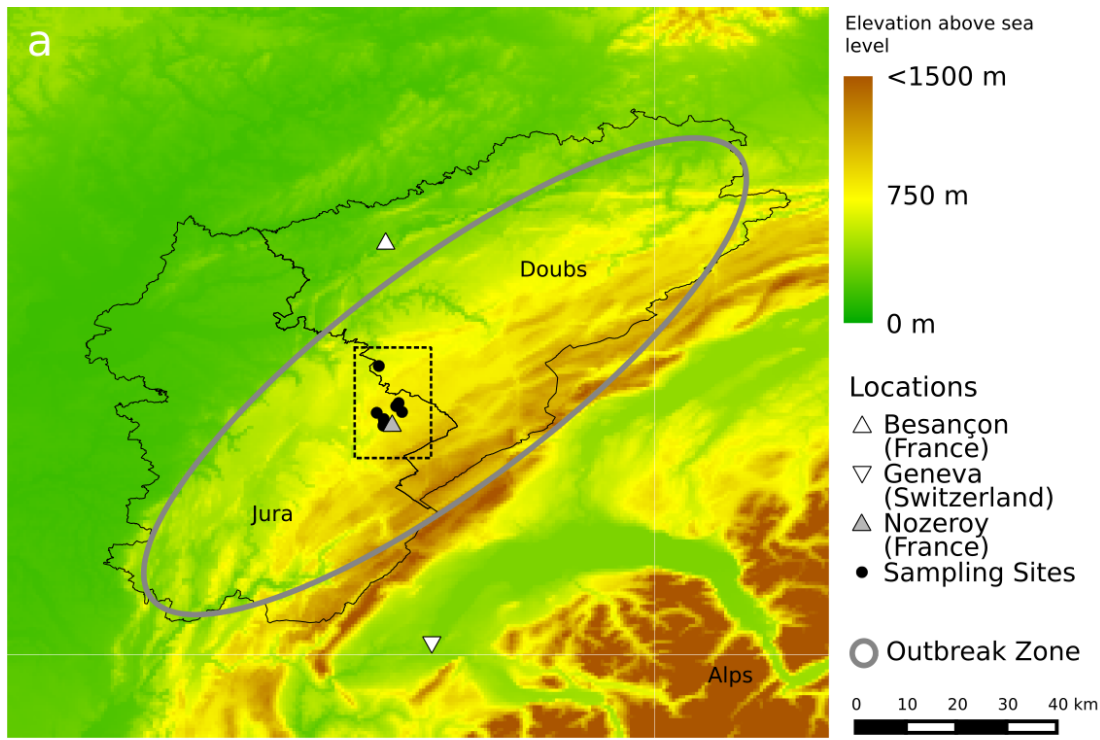
# Study Sites and Molecular Analysis

## 2.1 Study Sites

Five study sites were used to address the thesis objectives described in Chapter 1; each study site is located in a different commune in the Franche-Comté region of eastern France. This region is characterized by a low-elevation plain (200-400 m above sea level), a series of plateaus (400-700 m and 700-900 m in elevation) and the Jura mountains (900-1400 m in elevation, Figure 2.1.a). This region is a mix of managed forest and agricultural land (permanent grassland mowed for hay production, pasture in which cattle are grazed, or cultivated cereal). Three of the five communes (Cuvier, Doye and Onglières) were selected because they were sampled for *A. terrestris* in 2003-2005 (Berthier et al., 2006; Charbonnel et al., 2008b) and tissue samples from those investigations were made available for our use. These three sites are located in one zone of continuous grassland, centered around the village of Nozeroy in the Jura department (Table 2.1, Figure 2.1.b). The remaining two sites, Censeau (also in the Nozeroy grassland zone), and Arc-sous-Montenot (15 km north of Onglières in the Doubs department and separated from the other 4 sites by forest) were selected because at the time this work was started (Autumn 2014), local *A. terrestris* abundances in Arc-sous-Montenot and Censeau were very different and we wanted to compare bacterial parasite communities at different host abundances.

**Tab. 2.1.:** Sampling location coordinates. The communes of Cuvier, Doye and Onglières were sampled in 2003-2005 and in 2015-2016; 2015-2016 sampling in Onglières could not be conducted in the same location as in 2003-2005 due to landscape changes (grassland is now scrub); the 2015-2016 sampling site is within 2 km of the 2003-2005 sampling site.

Period	Location	Code	Position
2003-2005	Cuvier	CU	46.817 N, 6.050 E
	Doye	DY	46.767 N, 6.017 E
	Onglières	NG	46.802 N, 6.002 E
2014-2016	Cuvier	CU	46.824 N, 6.056 E
	Doye	DY	46.774 N, 6.018 E
	Onglières	NG	46.784 N, 6.018 E
	Arc-sous-Montenot	AM	46.925 N, 6.005 E
	Censeau	CE	46.802 N, 6.065 E



**Fig. 2.1.:** The study area. a) An elevation map of the Doubs and Jura departments in eastern France. The area in which *Arvicola terrestris* outbreaks occurs is indicated with a grey ellipse, and encompasses a plateau-mountain system ranging in altitude from 400-1400 m. The dotted box indicates the area shown in b) the historical (2003-2005) and current (2015-2016) sampling locations, with photographs for Arc-sous-Montenot, Cuvier and Doye. Sources: Topography is from GTOPO30, data available from the U.S. Geological Survey. Department borders are from OpenStreetMap ©OpenStreetMap contributors, data available under the Open Database License. Satellite image from Google maps, ©Google, Landsat/Copernicus, DigitalGlobe. Photo credits: Petra Villette.

**Tab. 2.2.:** Sampling session dates and the number of animals collected (males, females).

Date	Arc-sous -Montenot	Censeau	Cuvier	Doye	Onglières
2003 - Spring	-	-	16 (7,9)	16 (8,8)	-
2003 - Autumn	-	-	29 (14,15)	19 (10,9)	22 (9,13)
2004 - Spring	-	-	31 (16,15)	18 (8,10)	19 (8,11)
2004 - Autumn	-	-	18 (9,9)	21 (12,9)	16 (9,7)
2005 - Spring	-	-	-	14 (8,6)	21 (7,14)
2014 - Autumn	35 (11,24)	31 (12,19)	-	-	-
2015 – Spring	31 (15,16)	33 (22,11)	34 (18,16)	31 (16,15)	34 (19,15)
2015 – Autumn	42 (19,23)	33 (11,22)	34 (8,26)	30 (13,17)	35 (16,19)
2016 – Spring	30 (10,20)	31(17,16)	37 (16,21)	31 (14,17)	30 (16,14)

### 2.1.1 Animal Trapping and Dissection

Krebs (2013) has argued that a biologically relevant time step for sampling rodents is on the order of several weeks due to their fast life histories, but destructive sampling (i.e. removing animals from the population) at that frequency is likely to alter the population dynamics we wish to observe (Krebs et al., 1978), and would be prohibitively expensive and time consuming. Thus, sampling sessions were conducted in the spring and autumn of 2003-2005 and 2014-2016 (Table 2.2), and we limit ourselves to inferences based on broad time scales.

Sampling in 2003-2005 was conducted under the direction of Dr. Nathalie Charbonnel of the Centre de Biologie pour la Gestion des Populations (Montpellier) in Cuvier (CU), Doye (DY), and Onglières (NG) in April and October using live traps spaced to sample different burrow systems; traps were set between 11 am and 3 pm and checked every 90 minutes. Captured animals were taken to the lab for an immunological experiment before being euthanized via cervical dislocation and dissected (Charbonnel et al., 2008b). Animals were sexed and weighed to the nearest 0.1 g, and their spleens were stored in RNAlater and frozen at -80C.

Current sampling was conducted in Arc-sous-Montenot (AM), Censeau (CE), Cuvier (CU), Doye (DY), and Onglières (NG). We found landscape changes and a lack of *A. terrestris* sign in some of the historical sampling sites when we surveyed these sites in spring 2015, so current sampling was conducted in different parcels within the same communes (within 2 km of the historical sites). We conducted sampling sessions of 2 days duration in April-May and November-December of 2015 and 2016 at all five sites, and also in November of 2014 in AM and CE. We set unbaited Sherman traps (H.B. Sherman Traps Inc., Florida, USA) between 8:30 and 9:30 in the morning, and checked them no more than 2 hours later, for a maximum of five checks during a day, after which we removed all traps (Figure 2.2). Traps were placed 50-60 m apart to avoid trapping





**Fig. 2.2.:** Trapping *Arvicola terrestris* in grassland. a) *Arvicola terrestris* tumuli; tumuli are approx. 20cm in diameter and 5-10cm in height. b) A fresh tumulus (soil is moist and crumbly, no vegetation sprouting in it) is selected and dug up, exposing one or more tunnels. c) an unbaited Sherman trap is placed in the hole, aligned with one of the exposed tunnels. Traps were marked with a unique alphanumeric code, and their GPS locations were recorded.

the same burrow system twice and to ensure that sampling at high and low abundances was spatially similar. Two days of trapping at a site were sufficient for capturing at least 30 individuals.

Trapped animals were dissected on site following Auffray et al. (2011) (Figure 2.3). One at a time, individuals were euthanized via cervical dislocation and at least 0.5 mL of blood was collected via cardiac puncture with a syringe. Animals were weighed and sexed, and reproductive characteristics were recorded: testicle position (scrotal or abdominal) and testes length and width for males, nipples (lactating or not lactating), vagina (open or closed), pubic symphysis (open, slightly open, or closed), and number of embryos or placental scars for females. Males were considered reproductively active if their testes were scrotal, and females were considered reproductively active if they carried embryos or were lactating.

For each animal, the heart and portions of the liver, lungs, kidneys, and spleen were stored in RNAlater and frozen within 36 hours of collection. Dissection tools were sterilized with bleach, water and alcohol between animals, but not between organs. We do not think that cross contamination between organs during the dissection process is significant because when we performed a pilot study to determine if different organs



**Fig. 2.3.:** Dissecting *Arvicola terrestris* in the field. a) The portable tent set up at each sampling location during the sampling session. b) An *Arvicola terrestris* adult at the start of the dissection. c) The dissection table, organized to accommodate two simultaneous dissections and recording of meta data (animal weight, sex etc.)



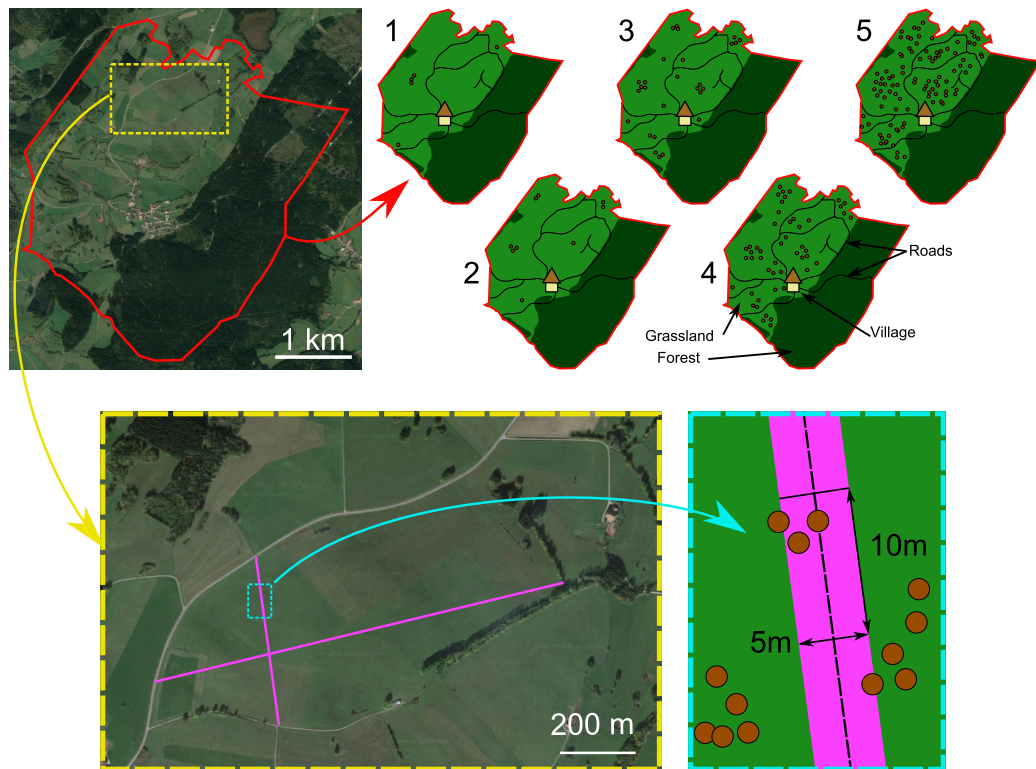
collected from the same animal host different bacterial species, we found that bacterial community composition differs significantly between organs collected from the same animal even when dissection tools are not sterilized between organs (see Box 2.1 for details).

Eyes were removed and stored whole in 75 % alcohol at 4 °C until they could be weighed; the crystalline lenses were removed from the eye tissue and allowed to dry at 45-55 °C for at least 5 days before being weighed to the nearest 1/10 mg. Lens weights hereafter refers to the pooled weights of the two lenses from each animal; in instances where only one lens could be weighed (the animal was missing an eye or the lens was destroyed during dissection), lens weight for that animal was simply double the weight of the single lens. In instances where the two lens weights differed by more than 50% of their mean (i.e. the smaller lens was less than one third the mass of the larger lens), the larger of the two lenses was doubled (this was applied to 51 animals out of 499).

### 2.1.2 Abundance Indices

We assessed local *A. terrestris* relative abundance during each sampling session using the transect method described in Giraudoux et al. (1995); 5 m-wide transects crossing the parcels from which animals were collected were divided into intervals 5 m long (2003-2005) or 10 m long (2014-2016) and the proportion of intervals positive for fresh tumuli was taken as an index of abundance (Figure 2.4).

Abundance was also assessed at the commune-scale by technicians of the FREDON-FC (Fédération Régionale de Défense controles Organismes Nuisibles de Franche-Comté) each season of 2014-2016 except for spring 2016, in which only Arc-sous-Montenot was assessed. The FREDON assessment uses a ranking system shown graphically in Figure 2.4 that ranges from 0 to 5: 0 - no *A. terrestris* sign in any parcel within the commune; 1 - low or no *A. terrestris* tumuli, voles and moles (*Talpa europaea*) cohabiting the same tunnel systems; 2 - *A. terrestris* tumuli present in some parcels within the commune, mole burrow systems still present in some parcels; 3 - *A. terrestris* tumuli present in some parcels within the commune, few or no mole burrow systems present in the commune; 4 - *A. terrestris* colonies established in the majority of meadows and within pastures; 5 - all of the commune is colonized by *A. terrestris*. The FREDON index does not directly translate to transect based indices, partly because it is applied at the commune scale and not the parcel scale, but Giraudoux et al. (1995) have shown that levels 0-1 correspond to densities < 100 animals/ha, level 2 to 100-200 animals/ha, and levels 3-5 to > 200 animals/ha. It must be emphasized that local and commune-scale abundances may differ dramatically, given that an individual parcel may have few *A. terrestris* but the commune as a whole is well infested, and vice versa.



**Fig. 2.4.:** Estimating abundance of *Arvicola terrestris* at the commune and parcel scale. *A. terrestris* colonies are represented as brown circles (not to scale). a. The commune of Arc-sous-Montenot (outlined in red) is assessed by vehicle on a scale of 0 to 5 (0 not shown). 0 - no *A. terrestris* sign in any parcel within the commune. 1 - low or no *A. terrestris* tumuli, voles and moles (*Talpa europaea*) cohabiting the same tunnel systems, 2 - *A. terrestris* tumuli present in some parcels within the commune, mole burrow systems still present in some parcels, 3 - *A. terrestris* tumuli present in some parcels within the commune, few or no mole burrow systems present in the commune, 4 - *A. terrestris* colonies established in the majority of meadows and within pastures, 5 - all of the commune is colonized by *A. terrestris*. Based on Figure 19.6 of Delattre and Giraudoux (2009) b. A parcel in Arc-sous-Montenot (yellow box in A) is assessed by foot using the transect method described in Giraudoux et al. (1995). 5-metre-wide transect (violet lines) are divided into 10-metre-long intervals; if an interval contains fresh *A. terrestris* tumuli, the interval is recorded as positive for *A. terrestris*. The proportion of intervals in the transect positive for *A. terrestris* is taken as an index of abundance.

## 2.2 Identifying Bacterial Parasites

To address the thesis objectives presented in Chapter 1, we used high-throughput DNA sequencing of the 16S rRNA bacterial gene to identify bacterial parasites present in the tissues of *A. terrestris* collected historically and currently. For the purposes of this thesis we adopt terminology modified from the Princeton Guide to Ecology's chapter on Ecological Epidemiology (Begon, 2009): a parasite is "an organism that obtains its nutrients from one or a very few host individuals, normally causing harm but not causing death immediately", and a pathogen is "any parasite that gives rise to a disease". We further restrict our definition of bacterial parasites to those bacteria whose survival or reproduction outside of a host is limited; in this way we exclude bacteria that are known to cause infection in immunocompromised individuals but are often found as active components of environmental microbial communities (e.g. *Acinetobacter baumannii* is an opportunist pathogen in hospitals and veterinary clinics (Francey et al., 2000), but is found globally in water and soil and as such would not be called a parasite under our definition).

We also use terminology adapted from Poulin (1995); Poulin defines a parasite community as all of the individuals of all parasite species found within a host population, and a parasite infracommunity as all individuals of all parasite species infecting an individual host.

Lastly, we have used the term bacterial species above and throughout this thesis but in practise the taxonomic level we use is the Operational Taxonomic Unit, or OTU. The use of the OTU stems from the following problem: the most robust bacterial taxonomies are based on what is known of cultured organisms, but molecular methods like 16S rRNA gene sequencing can detect rare bacterial taxa or difficult-to-culture bacterial taxa that are thus not well defined taxonomically (Schloss and Westcott, 2011). Thus, molecular ecologists face the challenge of binning sequences into microbial populations when in many cases they have little to no information about the degree of genetic variation within the population. Two approaches can be used for binning sequences, the phylotype-based approach and the OTU-based approach. The phylotype-based approach involves classifying sequences into bins based on their similarity to a reference taxonomy (e.g. Wang et al., 2007), which relies on a well-defined taxonomy that for difficult-to-culture or candidate taxa may not exist. The OTU approach involves classifying sequences into bins based on their similarity to each other (e.g. Schloss and Handelsman, 2005), and not a reference taxonomy; in this method, all sequences in the dataset can be assigned to bins regardless of whether or not the sequence is represented within a reference taxonomy. We use the OTU method here, with a 97 % similarity threshold for binning sequences together; this threshold is often used (e.g. Galan et al., 2016; Diagne et al., 2017; Jesus et al., 2009) but is somewhat controversial (Konstantinidis et al., 2006).

The work flow, from trapping the animals in the field to the generation of a read abundance table in which the number of sequence reads for each Operational Taxonomic Unit in each tissue sample is listed, is shown in flowchart form in Figure 2.5. The tissues from 825 animals were analyzed: spleens from 260 animals collected from the historical period (2003-2005) and spleens and mixes of heart, lung, liver, and kidneys from 565 animals collected from the current period (2014-2016). Processing these samples using the protocols developed by Kozich et al. (2013) and Galan et al. (2016) (detailed below) required that we group samples into four sequencing "runs" or batches which were processed separately until the bioinformatics step when they were analyzed together. Negative and positive controls within each run were used to filter reagent contaminants and cross contaminants from their respective runs, then OTU selection was applied to all runs together to generate one read abundance table with the cleaned, binned, and filtered sequences from all four runs.

### 2.2.1 DNA Extraction

Bacterial parasites may target one or more organs during infection (e.g. *Leptospira* sp. targets the kidney; Adler and de la Peña Moctezuma 2010), and in general the spleen is an important component of the host immune system that clears bacteria from the blood (Altamura et al., 2001), but how these phenomena might influence the resulting bacterial communities detected with PRC and sequencing is unclear. To address the uncertainty in the effect organ choice has on the identity of bacteria detected in *A. terrestris*, we conducted a small-scale study of the bacterial communities detected in the spleen, liver, heart, lungs and kidneys of 13 male *A. terrestris* from two of our study sites. The results of this study were published (Villette et al., 2017), the full text can be found at page 37. The results of the study are briefly presented in Box 2.1, and our general conclusion is that bacterial communities differ significantly between organs, and bacterial detection in *A. terrestris* is maximized when all five organs are included. But due to the number of animals included in this analysis, sequencing the bacterial communities of the heart, liver, lung, kidneys and spleen of each animal individually was unfeasible. Pooling all five organs together would limit our ability to compare the animals collected in 2014-2016 to those collected historically in 2003-2005, as only spleens from historical animals were used. To maximize the number of animals analyzed while retaining the ability to compare across historical and current sampling sessions, we pooled the heart, lung, liver and kidney tissues for each animal from 2014-2016 during the first step of DNA extraction, and extracted spleen DNA separately.

**Box 2.1: Consequences of organ choice in describing bacterial pathogen assemblages in a rodent population (Villette et al., 2017)**

High-throughput sequencing technologies now allow for rapid cost-effective surveys of multiple pathogens in many host species including rodents, but it is currently un-

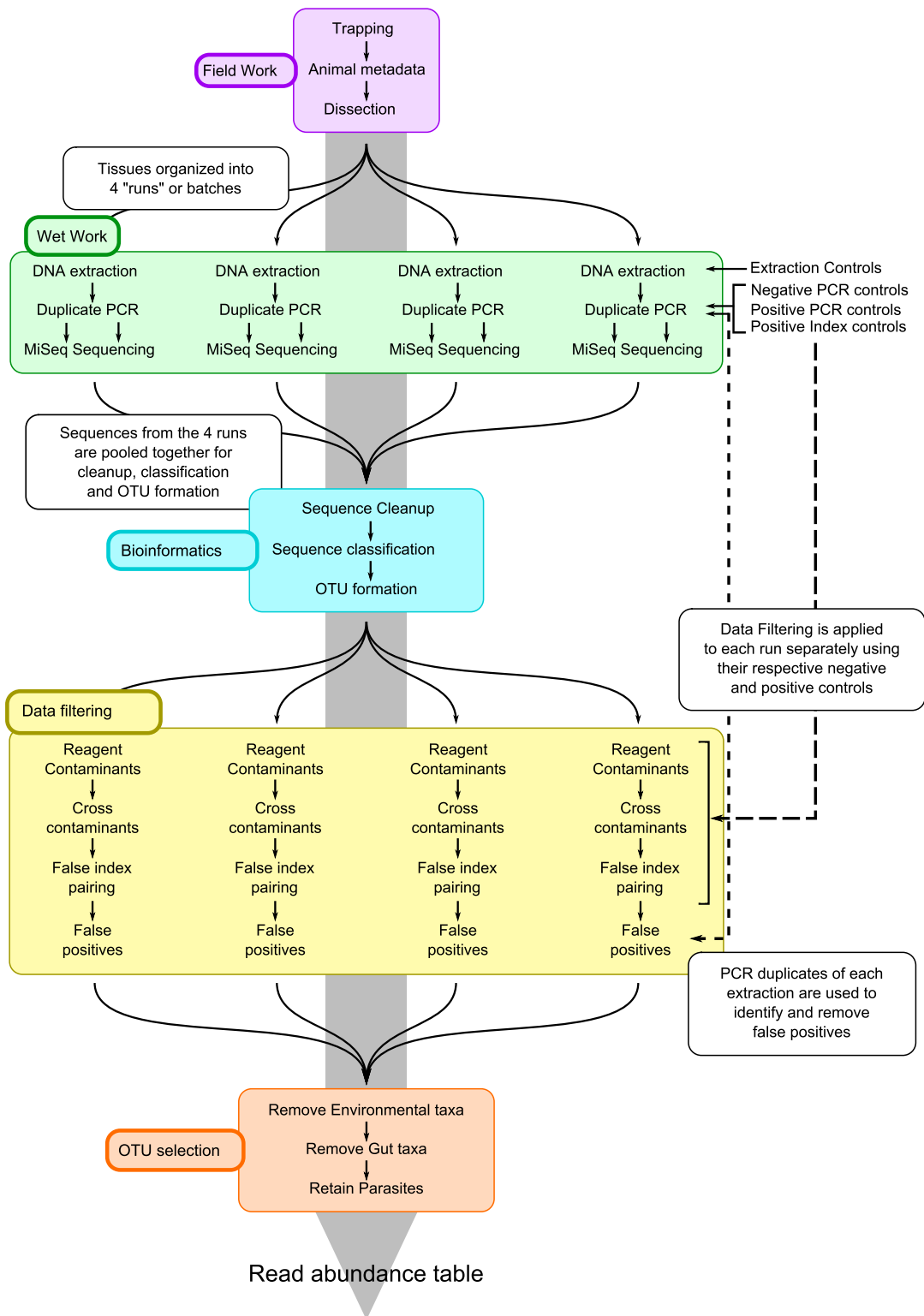


Fig. 2.5.: Flowchart of the work flow used to process tissue samples.

clear if the organ chosen for screening influences the number and identity of bacteria detected. We used 16S rRNA amplicon sequencing to identify bacterial pathogens in the heart, liver, lungs, kidneys and spleen of 13 water voles (*Arvicola terrestris*) collected in Franche-Comté, France. We asked if bacterial pathogen assemblages within organs are similar and if all five organs are necessary to detect all of the bacteria present in an individual animal. We identified 24 bacteria representing 17 genera; average bacterial richness for each organ ranged from  $1.5 \pm 0.4$  (mean  $\pm$  standard error) to  $2.5 \pm 0.4$  bacteria/organ and did not differ significantly between organs. The average bacterial richness when organ assemblages were pooled within animals was  $4.7 \pm 0.6$  bacteria/animal; Operational Taxonomic Unit accumulation analysis indicates that all five organs are required to obtain this. Organ type influences bacterial assemblage composition in a systematic way (PERMANOVA, 999 permutations, pseudo- $F_{4,51} = 1.37$ ,  $P = 0.001$ ). Our results demonstrate that the number of organs sampled influences the ability to detect bacterial pathogens, which can inform sampling decisions in public health and wildlife ecology.

We used the Qiagen DNeasy 96 kit which utilizes 96-well plates to process large numbers of samples concurrently. We included a negative control well in each plate in which no tissues were added. The tissues were lysed overnight using proteinase K, and DNA was extracted following kit instructions until the last step, in which DNA was eluted using 2 steps of 100  $\mu$ L of kit buffer AE to maximize DNA yield. DNA concentrations were measured using the NanoDrop 800 UV-Cis spectrophotometer (Thermo Fisher Scientific, France); DNA samples with concentrations less than 5 ng/ $\mu$ l were re-extracted using the Qiagen DNeasy blood and tissue kit (single tubes).

## 2.2.2 PCR Amplification and Sequencing

PCR amplification, indexing, multiplexing and demultiplexing followed Kozich et al. (2013) with modifications detailed by Galan et al. (2016). Negative controls and general procedures for removing biases and ensuring the quality of the data for later interpretation followed the guidelines in Table 1 of Galan et al. (2016); we used *Mycoplasma putrefaciens*, *Mycoplasma mycoides*, *Borrelia burgdorferi*, and *Bartonella taylorii*, for positive PCR amplification controls. We used primers 16S-V4F/16S-V4R dual-indexed with 8bp-indices (i5 and i7) and Nextera Illumina adapters to perform two PCR replicates for each sample in 5  $\mu$ L of 2  $\times$  Multiplex PCR Kit (Qiagen), 4  $\mu$ L of combined forward and reverse primers (2.5  $\mu$ M) and 2  $\mu$  L of DNA extract. The PCR program consisted of an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 20s, annealing at 55°C for 15s, and elongation at 72°C for 5 minutes, followed by a final extension step at 72°C for 10 minutes. PCR amplification was verified by electrophoresis in a 1.5 % agarose gel. Pooled amplicons were size-selected by excision after gel electrophoresis with 1.25% agarose gel in 1%

TAE buffer, and were purified using the NucleoSpin Gel clean-up kit (Machery-Nagel Sarl, France). We performed quantitative PCR with the KAPA library quantification kit (KAPA Biosystems) as per Galan et al. (2016) to quantify the DNA of the final library, and then loaded the library on the Illumina MiSeq flow cell using a 500 cycle reagent cartridge and 2 x 251bp paired-end sequencing, which yielded high-quality sequences through the reading of each nucleotide of the V4 fragments twice after the assembly of reads 1 and reads 2.

### 2.2.3 Bioinformatics and Data Filtering

Sequence analysis and taxonomic classification were performed using the mothur program package (Schloss et al., 2009) and following the standard operation procedure of Patrick D. Schloss (Kozich et al., 2013). All 4 sequencing runs were analyzed together. Briefly, reads 1 and 2 of each sample were assembled into contigs and long contigs (over 275 bp, or 24 bp longer than the target V4 region) or contigs with ambiguous bases were removed from the data set. Identical sequences were merged to reduce computation requirements. Sequences were aligned to the Silva SSU reference database v119 (Quast et al., 2013), filtered to remove poorly-aligned sequences, and trimmed to remove any columns in the alignment that have only gap characters; any identical sequences produced after these steps were merged to form unique sequences. Sequences were then sorted by abundance and compared to each other; sequences differing by less than 3 base pairs were merged. At this point sequences that appeared only once or twice in the data set were removed. Chimeras (two or more sequences joined together) were detected using the program UCHIME implemented in mothur; sequences flagged as chimeric in one sample were deleted from that sample but not any other sample. Sequences were classified using the Silva SSU reference database and sequences classified as belonging to chloroplasts, mitochondria, archaea, eukaryotes, and unknowns are removed. Sequences were then grouped into operational taxonomic units (OTUs) using a cutoff of 97 % similarity and a read-abundance table listing the number of reads of each resulting OTU in each sample was generated. Our *Mycoplasma* positive controls were assigned to Entomoplasmataceae rather than Mycoplasmataceae due to a frequent taxonomic error reflected in most databases, including Silva (Gasparich et al., 2004).

We filtered the read-abundance table following Galan et al. (2016). We used negative controls for both DNA extraction (1 well per 96-well extraction plate with reagents but no tissue) and PCR amplification (1 well per 96-well PCR plate with reagents but no DNA) to estimate cross-contamination rates for individual OTUs, and we used a false-assignment rate of 0.02 % to filter read abundances. To account for potential false-positives, we considered a tissue sample positive for a given OTU only if that OTU was present in both PCR replicates after the filtering steps described above were applied.

## 2.2.4 OTU selection

We then filtered the read abundance table further, removing OTUs that were typically found in soil or the digestive tract and retaining obligate bacterial parasites that have limited reproduction or survival outside of its animal host. We chose to remove gut bacteria and environmental bacteria because although gut and environmental bacteria can cause infection (see Box 2.2 for details), such infections are often opportunistic (occur in individuals that are strongly predisposed toward illness), and without some measure of health of the host or an immune response it is difficult to determine if the environmental or gut bacteria detected in an *A. terrestris* host has actually infected the animal or is a contaminant from the dissection or sequencing process. We used the 2nd edition of Bergey's Manual of Systematic Bacteriology, Volumes 1 to 5 (Boone et al., 2009; Brenner et al., 2005a; Brenner et al., 2005b; Krieg et al., 2010; Goodfellow et al., 2012), Pathogenesis of Bacterial Infections in Animals (Gyles et al., 2010) and a general search of the literature to identify parasitic bacteria and environmental and gut bacteria.

### **Box 2.2: Gut bacteria and environmental bacteria as opportunistic pathogens in animals**

Gut bacteria can translocate across the intestinal wall transcellularly through the enterocytes or via tight junctions, entering the mesenteric lymph system and the blood stream and causing infection in other organs. The majority of research into this phenomenon focuses on human cases (Balzan et al., 2007), but in mouse and rat models, translocation has been observed in association with underlying conditions like an absence of gut flora, or overgrowth of particular taxa in individuals with a normal gut flora (Berg and Garlington, 1979).

Nutrition also appears to be important in bacterial translocations; Deitch and Berg (1987) found that in specific pathogen-free mice bacterial translocations occurred in 80 % of mice within 24 hours of being administered endotoxin, but this incidence of translocation was higher when the mice were also protein-malnourished, with higher numbers of bacteria being observed in organs as well.

Some environmental bacteria are well-known opportunistic pathogens; for example, *Acinetobacter baumannii* is a well-known cause of infection of immunocompromised animals and people and a serious problem in hospitals and veterinary clinics worldwide (Francey et al., 2000; Bergogne-Berezin and Towner, 1996), but it's also a common component of soil microbial communities globally. *Pseudomonas aeruginosa* is another opportunistic infection of humans and other animals that is also found in water and soil (Westman et al., 2010). Because these organisms are opportunistic infections, their detection in our data set may be due to their infecting hosts, but it could also be due to inadvertent soil contamination during the dissection process.





## SHORT REPORT

### Consequences of organ choice in describing bacterial pathogen assemblages in a rodent population

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Received 17 January 2017; Final revision 19 May 2017; Accepted 3 August 2017

#### SUMMARY

High-throughput sequencing technologies now allow for rapid cost-effective surveys of multiple pathogens in many host species including rodents, but it is currently unclear if the organ chosen for screening influences the number and identity of bacteria detected. We used 16S rRNA amplicon sequencing to identify bacterial pathogens in the heart, liver, lungs, kidneys and spleen of 13 water voles (*Arvicola terrestris*) collected in Franche-Comté, France. We asked if bacterial pathogen assemblages within organs are similar and if all five organs are necessary to detect all of the bacteria present in an individual animal. We identified 24 bacteria representing 17 genera; average bacterial richness for each organ ranged from  $1.5 \pm 0.4$  (mean  $\pm$  standard error) to  $2.5 \pm 0.4$  bacteria/organ and did not differ significantly between organs. The average bacterial richness when organ assemblages were pooled within animals was  $4.7 \pm 0.6$  bacteria/animal; Operational Taxonomic Unit accumulation analysis indicates that all five organs are required to obtain this. Organ type influences bacterial assemblage composition in a systematic way (PERMANOVA, 999 permutations, pseudo- $F_{4,51} = 1.37$ ,  $P = 0.001$ ). Our results demonstrate that the number of organs sampled influences the ability to detect bacterial pathogens, which can inform sampling decisions in public health and wildlife ecology.

**Key words:** *Arvicola terrestris*, High-Throughput Sequencing, bacterial pathogens, rodent-borne pathogen, tissue selection.

Gaps in our understanding of bacterial species distributions, functions and communities are of particular interest within wildlife ecology because pathogens (bacterial or otherwise) have the potential to regulate

host populations [1], which can in turn drive ecosystem dynamics. In addition, zoonoses pose a significant human health risk in much of the world, accounting for 60% of emerging infectious diseases documented since 1940, 70% of which originated in wildlife [2].

Both of these concerns intersect in rodents. Several species of vole and lemming in Europe and North America, including the fossorial water vole *Arvicola terrestris* in France, exhibit multi-annual population cycles characterized by explosive population growth

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followed by rapid catastrophic population declines [3]. These cycles are not completely understood, and pathogens are one potential driver of these cycles that has yet to be fully explored. Rodents can also be reservoir species for zoonotic bacteria around the world [4].

The development of high-throughput sequencing and 16S rRNA amplicon sequencing approaches, which allows for the simultaneous identification of multiple bacterial species in large sample sizes, now allows for the study of bacterial communities within hosts and host populations [5, 6], but methodological questions still remain. For example, tissue selection within hosts is one area that has received little attention. Comparing multiple organ tissues within the same rodent has been done before for single bacterial species [7, 8], but to our knowledge, the present study is the first to survey multiple organs for their bacterial assemblages.

Our objectives were the following: first, describe the bacterial pathogens present in 13 *A. terrestris* heart, liver, lung, kidney and spleen tissues. Currently little is known about the bacteria present in this species in the Franche-Comté region of eastern France. Our second objective was to compare bacterial assemblages among organs. We hypothesized that bacterial assemblages would differ in richness and membership among tissue types because the five organs we have targeted differ in both function and potential exposure routes. We were also interested in determining how many organ types are required to obtain complete coverage of the total bacterial assemblage of the five organs. We hypothesized that there would not be complete species turnover between organs, and that a subset of organs would contain the majority of bacterial pathogens found in the pooled assemblage.

We conducted sampling in the communes of Arc-sous-Montenot (46°25' N, 6°05' E) and Censeau (46°02' N, 6°06' E) in the Doubs and Jura departments of eastern France in November of 2014. The two sampling locations are approximately 14 km apart, and separated by a mix of pasture, cultivated agricultural land and forest. Population abundance at the two sites was assessed by at least 1.5 km of 5 m-wide transects [9]; the proportion of 10 m intervals within the transects positive for recent vole activity (tumuli) were 91% and 7% in Arc-sous-Montenot and Censeau, respectively. We collected nine adult males from Arc-sous-Montenot and four adult males from Censeau as part of a larger sampling program (metadata available through the

dat@osu platform [10]). We trapped the animals using unbaited Sherman live traps spaced at least 10 m apart, and euthanized them by cervical dislocation. The 13 animals' weight ranged from 75 g and 97 g (mean 84.3 g), and none of the animals were reproductively active at time of collection (testes were retracted into the abdomen). Portions of the heart, lungs, liver, spleen and kidneys were stored individually in 2 ml tubes of RNAlater (Thermo Fisher Scientific, Illkirch-Graffenstaden France) and subsequently frozen at -20 °C. To prevent cross-contamination between animals during dissection, we systematically alternated the use of two sets of dissecting instruments, which were decontaminated using bleach, water and 95% ethanol between animals [11]. We did not decontaminate dissecting instruments between organs within animals, and therefore did not eliminate the possibility of cross-contamination between organs within animals. Animals were treated in accordance with European Union guidelines and legislation (Directive 86/609/EEC). The Chrono-environment laboratory received approval from the *Comité d'Ethique Bisontin en Expérimentation Animale* (CEBEA N°58) for the sampling of rodents and the storage and use of their tissues. The rodent species investigated in this study does not have protected status (see IUCN and CITES lists), and is listed as a pest, subject to control, under Article L201-1 of the *Code Rural et de la Pêche Maritime* of French law.

We extracted DNA from 65 tissue samples (13 individuals × 1 tissue sample for each of the five organs collected per animal) using the DNeasy® 96 tissue kit (Qiagen, Hilden, Germany) following the manufacturer instructions. We measured DNA concentration for each sample using a NanoDrop 8000 UV-Cis spectrophotometer (Thermo Fisher Scientific, France); all samples had a DNA concentration higher than 10 ng/μl.

We sequenced a 251 bp fragment of the V4 region of the 16S rRNA gene following Kozich *et al.*'s procedure [12] with modifications detailed by Galan *et al.* [6] for PCR amplification, indexing, multiplexing and demultiplexing and taxonomic identification using the SILVA SSU database as a reference (see Supplementary Material 1, available on the Cambridge Core website, for details). Sequences were grouped into Operational Taxonomic Units (OTUs) using a 97% similarity threshold, and only those OTUs identified as potentially pathogenic were included in the subsequent analysis (see Supplementary Material 1 for details). In the rest of

this article, the terms ‘OTU’ and ‘bacteria’ are used to describe potentially pathogenic agents only.

All statistics were conducted using R (version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria). We calculated the average number of OTUs detected in each tissue sample (OTU richness) as well as the average OTU richness in each animal when tissues were pooled together. We used the `glmer` function in the `lme4` package [13] to fit a generalized linear mixed model to determine if OTU richness differs between organs. We used a Poisson distribution with log link function, animals nested in locations were included as random factors, and the model was fit using maximum likelihood (Laplace approximation). To determine if pooling organ data significantly increased OTU richness, we used bootstrapping (999 iterations) to estimate the average difference (with 95% confidence intervals) between the heart and pooled OTU richness within animals.

We calculated Jaccard’s presence/absence dissimilarity indices between each organ within animals to quantify bacterial assemblage differences, and we used the `adonis` function in the `vegan` package [14] to perform a PERMANOVA (permutational multivariate analysis of variance) on the Jaccard dissimilarities to determine if bacterial assemblages within organs were non-random. We stratified organs by animal to account for any between-animal variation, and included a location term and interaction term between location and organ as well. We used the `betadisper` function in the `vegan` package to check for homogeneity of dispersions of the groups, and set the number of permutations to 999. We also performed a PERMANOVA analysis to determine if bacterial assemblages differ between animals (see Supplementary Material 4 for details).

We performed an OTU accumulation analysis to assess the effect of including more than one organ in calculating animal OTU richness. Organs were included in the OTU richness calculation for each animal in order of decreasing average OTU richness (first the heart, then the kidneys, then the spleen, then the liver, then the lungs). Means and 95% confidence intervals were estimated via bootstrapping (999 iterations).

A total of 24 OTUs representing at least 17 genera were included in the final analysis (Table 1). One OTU could not be assigned to a genus, its family classification (*Pasteurellaceae*) is listed instead.

Prevalence of individual OTUs ranged from 2% to 37% of all 65 samples, with prevalence within an organ ranging from 0% to 54% (Table 1). Average OTU richness within each organ ranged from  $1.5 \pm$

$0.4$  (mean  $\pm$  standard error) to  $2.5 \pm 0.4$  OTUs/organ (Table 2) and in general did not differ significantly between organs with the exception of the average lung OTU richness, which is marginally significantly lower than the average heart OTU richness (Table 3). Location did not account for any variance in the model, and was discarded. When pooling organs together within animals, average OTU richness was  $4.7 \pm 0.6$  OTUs, which is  $2.15 \pm 0.84$  OTUs (bootstrapped mean and 95% confidence interval) greater than the average OTU richness of the heart.

We determined that bacterial assemblages differ between organs (PERMANOVA, pseudo- $F_{4,51} = 1.37$ ,  $P = 0.001$ ) and between locations (pseudo- $F_{1,51} = 11.77$ ,  $P = 0.003$ ). The organ–location interaction term was also significant (pseudo- $F_{4,51} = 1.29$ ,  $P = 0.027$ ), suggesting that location effects are not the same across organs (see Supplementary Material 3 for additional analysis).

Adding organs increased the average OTU richness of each animal (Fig. 1); adding the kidney increased the average animal-level OTU richness by one OTU, and the addition of the spleen increased the average OTU richness by 0.8 OTU. Including the least-rich organs last, the liver and lung, still increased average OTU richness by 0.2 OTUs each.

We failed to detect differences in average OTU richness between organs, but we determined that bacterial assemblages vary between organs within individuals; if all of the organs within an animal had the same bacterial assemblages, we would expect the OTU accumulation analysis to show no increase in average OTU richness with increasing number of organs. This result is further supported by our PERMANOVA results, as differences in assemblages do appear to be mediated by organ type. This suggests that a holistic approach is better suited to surveying bacteria in this species, as bacterial assemblages within different organs within the same animal vary in both richness and membership. These results also suggest that cross-contamination between organs during the dissection and collection process was not significant; if significant cross-contamination had occurred, we would expect bacterial assemblages obtained from different organs within an animal to be similar.

The differences we detected in bacterial assemblages within organs may be due in part by spatial and temporal dynamics of bacterial infections. Deng *et al.* [15] report that while *Bartonella* infections result in high *Bartonella* densities in the spleen, *Bartonella* in the liver is detectable only early in the infection and

Table 1. OTU genera included in the analysis, and their prevalences (expressed as a percentage in all samples and each organ)

Genera	Prevalence					
	All organs n = 65	Spleen n = 13	Kidney n = 13	Heart n = 13	Lung n = 13	Liver n = 13
<i>Mycoplasma</i> (1) <sup>a</sup>	36.9	30.8	30.8	46.2	46.2	30.8
<i>Mycoplasma</i> (2) <sup>a</sup>	36.9	30.8	30.8	46.2	46.2	30.8
<i>Acinetobacter</i>	26.2	23.1	23.1	53.8	7.7	23.1
<i>Bartonella</i>	12.3	30.8	0	7.7	15.4	7.7
<i>Treponema</i>	12.3	7.7	23.1	15.4	0	15
<i>Avibacterium</i>	9.2	7.7	0	7.7	15.4	15.4
<i>Treponema</i>	9.2	7.7	23.1	0	0	15.4
<i>Pasteurellaceae</i>	7.7	15.4	0	7.7	7.7	7.7
<i>Helicobacter</i>	6.2	0	0	30.8	0	0
<i>Leptospira</i>	6.2	7.7	23.1	0	0	0
<i>Prevotella</i>	4.6	7.7	7.7	0	0	7.7
<i>Helicobacter</i>	3.1	0	7.7	0	0	7.7
<i>Aerococcus</i>	1.5	7.7	0	0	0	0
<i>Bartonella</i>	1.5	7.7	0	0	0	0
<i>Chryseobacterium</i> (1) <sup>b</sup>	1.5	0	0	7.7	0	0
<i>Chryseobacterium</i> (2) <sup>b</sup>	1.5	0	0	7.7	0	0
<i>Corynebacterium</i>	1.5	7.7	0	0	0	0
<i>Helicobacter</i>	1.5	0	0	0	7.7	0
<i>Leptospira</i>	1.5	0	7.7	0	0	0
<i>Peptococcus</i>	1.5	0	7.7	0	0	0
<i>Sphingomonas</i>	1.5	0	0	7.7	0	0
<i>Streptococcus</i>	1.5	0	0	7.7	0	0
<i>Filobacterium</i>	1.5	0	0	7.7	0	0
<i>Ureaplasma</i>	1.5	0	0	0	0	7.7

One OTU could not be classified to genus level, and is instead designated by family.

<sup>a</sup> Two different OTUs.

<sup>b</sup> Two different OTUs.

Table 2. Average OTU richness and standard error for each organ, and for organs pooled within animals

Organ	Average OTU richness	Standard error
Heart	2.5	0.4
Kidney	2.0	0.3
Spleen	1.8	0.3
Liver	1.7	0.4
Lung	1.5	0.4
All organs	4.7	0.6

for a brief period of time, and similar infection dynamics have been observed for *Borrelia burgdorferi* in mammals [16]. In addition, infectious bacteria may target organ tissues but the spatial and numerical distribution of bacteria within host cells can vary with host. For example, *Salmonella* infections in mice can be characterized by either the widespread but low-abundant presence of bacteria within host

cells [17], or a more localized infection with few host cells harboring many bacteria [18]. This potential for different infection patterns at the cellular level may be of particular importance here as we did not homogenize our organs before taking tissue samples; the apparent increase in OTU richness with increasing number of organs may in fact be a consequence of resampling the same animal five times, rather than differences in assemblages within each tissue type.

Host immuno-competence, body condition and genetics can also contribute to infection dynamics [19, 20] and may drive some of the variation we observed between animals from the same population.

The conclusions presented here are of course limited by the small sample size; OTU accumulation analyses for individual organs (Supplementary Material 2) strongly suggest that if we were to include additional animals in our analysis, we would detect

Table 3. Parameter estimates (log scale) and standard errors for fixed effects and variance and standard deviation for random effects of a generalized linear mixed effects model for average OTU richness within organs

Effect		Coefficient	S.E.	z-value	P-value
Fixed	Heart	0.86	0.21	4.09	4.30E-05
	Liver	-0.41	0.27	-1.49	0.137
	Lung	-0.55	0.29	-1.94	0.053
	Spleen	-0.28	0.26	-1.06	0.291
	Kidney	-0.31	0.27	-1.20	0.231
Random		Variance	Standard deviation		
	Animal	0.15	0.39		

The model was fit using maximum likelihood, a Poisson distribution and log link function.

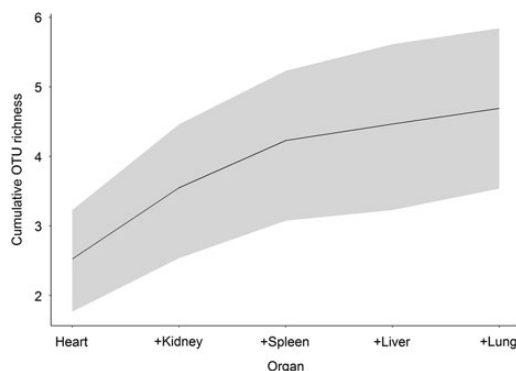


Fig. 1. Bootstrapped operational taxonomic unit (OTU) accumulation curve of the number of potentially pathogenic bacterial OTUs detected within 13 male *Arvicola terrestris* with increasing number of organs screened. Organs are ordered by average OTU richness, with the heart having the highest average OTU richness, and the lung having the lowest. The grey band is bootstrapped 95% confidence intervals (999 iterations). Animals were collected in Franche-Comté, France, in November 2014.

more OTUs. Additional tissues like lymph nodes, the brain, the digestive tract and skin lesions can harbor bacteria that may also be of interest, and experimenting with tissue homogenization and sub-sampling could provide insight into the results we obtained here. Determining if blood would yield results similar to the heart or other organs would also be of interest, given that all of the tissues we considered here are permeated by blood.

Our results demonstrate that the number of organs sampled influences the power to detect trends in bacterial pathogen assemblage composition. These results can inform sampling decisions in public health and wildlife ecology.

## SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at <https://doi.org/10.1017/S0950268817001893>.

## ACKNOWLEDGEMENTS

Data used in this work were partly produced through the genotyping and sequencing facilities of ISEM (Institut des Sciences de l'Évolution-Montpellier) and Labex Centre Méditerranéen Environnement Biodiversité. Analyses were performed on the Centre de Biologie pour la Gestion des Populations HPC computational platform thanks to Alexandre Dehne-Garcia, and on the supercomputer facilities of the Mésocentre de calcul de Franche-Comté. The authors would like to thank the residents of Arc-sous-Montenot and Censeau for their permission to sample in their communes, and Jean-Baptiste Pons for his assistance in the field. This research has been financially supported by the grant 2015-0011 FREDON-UFC and a PhD scholarship of the Région de Bourgogne Franche-Comté, and has been carried out within the framework of the Zone Atelier Arc Jurassien, <http://zaaj.univ-fcomte.fr>.

## DECLARATION OF INTEREST

None.

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# Parasitic Bacterial Communities of abundant *Arvicola terrestris* populations

## 3.1 Introduction

In Chapter 1 of this thesis we reviewed past research into the disease hypothesis of fluctuating rodent host populations and how parasite communities may influence and be influenced by their hosts. We outlined a broad goal of trying to understand how bacterial parasite communities change over the course of the outbreak of a cyclic rodent host, *A. terrestris*, but before we can understand changes during the decline phase, we must have an understanding of conditions during the peak phase, especially in regards to spatio-temporal variability in parasite communities at individual host and host population scales.

We adopt here a community ecology approach and focus on aspects of the parasite community, as opposed to the dynamics of individual parasite taxa. Early failures to detect a single parasite or pathogen as a causative agent of the decline phase of rodent cycles (see Chapter 1 details), as well as accumulating evidence that bacterial parasite communities are not random assemblages but in fact structured by a variety of mechanisms including interspecific interactions (e.g. Telfer et al., 2010; Vaumourin et al., 2014) makes it clear that to consider parasite species in isolation is to miss a significant part of the whole picture of host-parasite dynamics.

We use the terminology of Poulin and Morand (2004) with respect to scale; at the individual host scale we have the parasite infracommunity, or all of the parasites infecting one host. At the host population scale is the parasite component community, or all of the parasites infecting one host population. At the host meta-population scale we have the meta community, or all of the parasites found within the host meta population. The parasite fauna refers to all the parasites infecting the host species over the entirety of the host's range - we do not consider this scale here, and we also restrict ourselves to the bacterial parasite community, although it is likely that other parasitic organisms like viruses and helminths interact to shape the bacterial parasite communities in *A. terrestris* (Telfer et al., 2008).



We asked the following questions: first, how does parasite infracommunity richness and composition vary between *A. terrestris* hosts? Infracommunity richness is thought to be determined by the rate at which the host can be colonized by parasites and the duration of the subsequent infection (Poulin, 1995), with these two processes subject to extrinsic factors like location and time of year, and intrinsic factors like host age, density, sex, parasite virulence, etc. Much of the work on parasites in rodents has focused on extrinsic and intrinsic factors that determine risk of infection by, or prevalence of, a single parasite (e.g. Deter et al., 2007; Charbonnel et al., 2008a), but research regarding haemoparasites in Polish rodents found that extrinsic factors (season and year) were more important than intrinsic factors (sex and age) in determining infracommunity richness (Pawelczyk et al., 2004). Cerqueira et al. (2007) found that infracommunity richness of helminths in *A. terrestris* varied significantly over phases of the cycle, and in particular observed a statistically significant difference in richness between the early peak (abundance) and late peak (pre-decline phases) of the cycle. These results lead us to expect that infracommunity richness and composition similarity will vary across *A. terrestris* individuals.

Second, how does component community richness and similarity vary between sampling sites? At this scale, island biogeography and epidemiology have both been used to make predictions regarding parasite richness: island biogeography theory predicts that at equilibrium and when populations are distinct, component community richness will depend on host population size and isolation from other host populations (Kuris et al., 1980). Epidemiological models predict that high-density populations will host more parasites because they promote larger  $R_0$  values for parasites compared to low-density populations;  $R_0$  (basic reproductive number) is a measure of parasite invasiveness, is usually calculated as the ratio of host population density and parasite transmission to natural and parasite-induced host mortality, and when less than 1 the parasite will not persist in the population (Poulin and Morand, 2004). Both of these frameworks focus on equilibrium conditions, and, as Kuris et al. (1980) noted, the assumptions of island biogeography theory (for example, isolated host populations) are unlikely to be met in the real world. But, there is some evidence to suggest that island biogeography theory, despite its limitations, can be applied to host-parasite systems: Spurgin et al. (2012) reported a species-area relationship in blood pathogens of Berthelot's pipit (*Anthus berthelotii*) in the Canary islands, and Lopez et al. (2005) reported that high rates of inter-population migration between experimental host populations of flour beetles resulted in higher occupancy (number of populations in the meta population infected by parasites) by an ectoparasite than meta populations with low inter-population migration. How these frameworks can be applied to populations that fluctuate like *A. terrestris* in Franche-Comté is unclear.

To answer these questions, we use generalized linear modelling to identify predictors of bacterial parasite infra- and component community richness in *A. terrestris* individuals

collected from five communes in Franche-Comté in spring and autumn 2014-2016. We use PERMANOVA analysis of Jaccard distances to assess community composition similarity, and we also assess the correlation between Jaccard distances and geographic distances between individual *A. terrestris* and populations of *A. terrestris*.

## 3.2 Materials and Methods

Study sites, animal trapping and dissection, and molecular methods are described in Chapter 2 of this thesis. Briefly, the communes Arc-sous-Montenot (AM), Censeau (CE), Cuvier (CU), Doye (DY) and Onglières (NG) were visited in spring and autumn of 2015 and spring 2016; AM and CE were also visited in autumn 2014. A minimum of 30 *Arvicola terrestris* were captured in live traps and dissected on site; animals were weighed and reproductive status was noted (males were classified as reproductively active if their testes were scrotal, females were classified as reproductively active if they were lactating or were pregnant). Hearts, livers, lungs, spleens and kidneys were collected and stored in RNAlater. DNA was extracted from spleens and from a mix of heart, liver, lung, and kidneys from each animal, and the 16s rRNA bacterial gene was amplified via PCR with indexed primers to tag amplicons with a unique identifying pair of sequences. Tagged amplicons were sequenced using the MiSeq Illumina platform, and sequences were filtered for quality using the program mothur and the SOP developed by Patrick Schloss. Sequences were binned into Operational Taxonomic Units using a 97 % similarity cutoff and classified to the genus level using the Silva rRNA library as a reference database. The resulting read-abundance table was further filtered using negative and positive controls introduced at the DNA extraction and PCR amplification steps to remove reagent contaminants, and potential false positives were removed using 2 PCR replicates per DNA extract. The resulting OTUs were filtered to remove gut and environmental bacteria (some of which may be opportunistic pathogens, see Box 2.2, Chapter 2) to leave only those bacteria that are obligate parasites, whose survival and reproduction outside of a rodent host is minimal.

### 3.2.1 Statistical analysis

All statistical analyses were performed using R software (version 3.4.0, R Core Team). We calculated infracommunity richness for each animal by pooling organ OTU assemblages within animals together and counting the number of unique OTUs found within each animal (ie. if an OTU appeared in both the spleen and other organs of an animal, it was only counted once). We used the score test (Van den Broek, 1995) to determine if infracommunity richness data was zero-inflated (it was,  $S(\tilde{\beta}) = 13.7$ ,  $p < 0.001$ ), therefore, to identify predictors of infracommunity richness, we modelled infracommunity richness using a zero-inflated generalized linear model with a Poisson distribution

and log link for counts and a binomial distribution with logit link for zeros (zeroinfl function from package pscl). We included current transect-based abundance indices ( $t-ab_0$ ), site, sex, reproductive status, date (season and year), and FREDON abundance indices from the previous season (approx. 6 months prior,  $F-ab_{-6}$ ) and the previous year (12 months prior,  $F-ad_{-12}$ ). Current FREDON abundance indices and time-lagged local abundance indices could not be included because they were not available for all sampling sessions.

Model selection was performed manually with all combinations of predictor variables for the counts and zero components of the models (interaction terms were not included due to computational limitations); models with a maximum variance inflation factor (VIF) score, calculated using the vif function from package car, greater than 10 (indicating collinearity between at least two of the predictor variables) were excluded. We performed likelihood ratio tests (function lrtest of package lmer) between the two models with the lowest AIC scores and further-simplified models to determine if further simplification altered model fit.

Because lens weights were not available for animals collected in 2014, the same model selection procedure was conducted using just the data from just 2015 and 2016 with age class as an additional term. Lens weights were binned into 3 age classes due to non-linearity:  $< 5$  mg - juvenile, 5-6.9 mg - young adult,  $> 7$  mg old adult (from Cerqueira et al., 2006)

To determine if compositional similarity between infracommunity composition correlated with geographic distances between capture points of hosts, we calculated straight-line distances between trapping locations of animals with infracommunity richness greater than 1 using the rdist.earth function of package fields, and performed Mantel tests using the Spearman correlation coefficient with the resulting distance matrix and its respective Jaccard distance matrix between infracommunities. Distances and dissimilarities were stratified by sampling date (season and year) to restrict comparisons to animals that were captured at the "same" time (ie. within one month of each-other). A bonferroni correction was applied to our  $\alpha$  value to account for the four tests ( $\alpha = 0.0125$ ). We also used PERMANOVA to identify host and host population characteristics that may structure infracommunity composition; using the adonis2 function of package vegan, we included current local abundance ( $t-ab_0$ ), sex, date, FREDON abundance indices from the previous season and year ( $F-ab_{-6}$  and  $F-ad_{-12}$  respectively), and study site in a model with the complete data set; we used all of the above terms as well as age class in a model with just data from 2015-2016. PERMANOVAs were conducted with 1000 iterations and marginal tests. To visualize Jaccard distances, we used nMDS ordination in two dimensions with the nmDS function from the ecodist package.

Component community richness was estimated using the bootstrap estimator implemented in the `specpool` function of package `vegan`; animals were grouped by sampling session. We modelled bootstrapped component community richness of the whole data set using generalized linear modelling with the Gaussian family and identity link, with study site, current local abundance ( $t-ab_0$ ), FREDON abundance indices from the previous season and year ( $F-ab_{-6}$  and  $F-ad_{-12}$  respectively), and date (season and year) included as predictors. We also modelled bootstrap component community richness of data from 2015-2016 with mean lens weight as an additional predictor. Model selection was exhaustive, with each combination of predictors tested (interaction terms were not included). Models were selected based on  $\Delta AIC$  and maximum VIF scores; models with  $\Delta AIC > 2$  and maximum VIF scores greater than 10 were excluded. We used maximum likelihood tests to determine if further simplification of the resulting "best" models altered model fit.

To determine if component community composition similarities are correlated with geographic distances between host populations, we performed Mantel tests with the Pearson correlation coefficient on Jaccard distances between component communities from the same date and their respective geographical distance matrices; distance between sampling sites was calculated between centroids of trapping locations within each site using the `rdist.earth` function of package `fields`. We also conducted PERMANOVAs with the `adonis2` function on the complete data set and just data from 2015-2016 using current local abundance ( $t-ab_0$ ), date, FREDON abundance indices from the previous season and year ( $F-ab_{-6}$  and  $F-ad_{-12}$  respectively), site as predictors for both data sets and mean lens weight as well for the 2015-2016 data subset. We checked for differences in within-group dispersion of each factor using the `betadisper` function from package `ecodist` with ANOVA. Jaccard distances between component communities were visualized using nMDS ordination with the `nmfs` function of package `ecodist`.

Meta community richness was estimated using the bootstrap estimator implemented in function `specpool`; animals were grouped together by sampling date.

## 3.3 Results

### 3.3.1 Hosts and OTUs

A total of 565 animals were included in the analysis, with an average of 33.2 animals per sampling session (range: 30 to 42) and a mean sex ratio of 45 % male (range: 23 % to 67 %) (Table 3.1). Mean dry lens weight of a sampling session ranged from 4.8 mg to 8.1 mg and did not differ between sexes (pair-wise Mann-Whitney  $u$ -tests for each sampling session with a bonferroni correction such that  $\alpha = 0.05/17 = 0.003$ ). The

proportion of each population reproductively active (scrotal testes in males and lactation or embryos in females) ranged from 11 % to 97 % and in general a lower proportion of females was reproductively active than males.

Local abundance for each sampling session, as assessed by transect indices, ranged from 4 % to 100 % (Figure 3.1) while commune abundance as assessed by the FREDON during this period ranged from 2 to 5 and followed similar trends to our transect-based abundance indices, with the exception of Censeau in autumn 2014 and spring 2015.

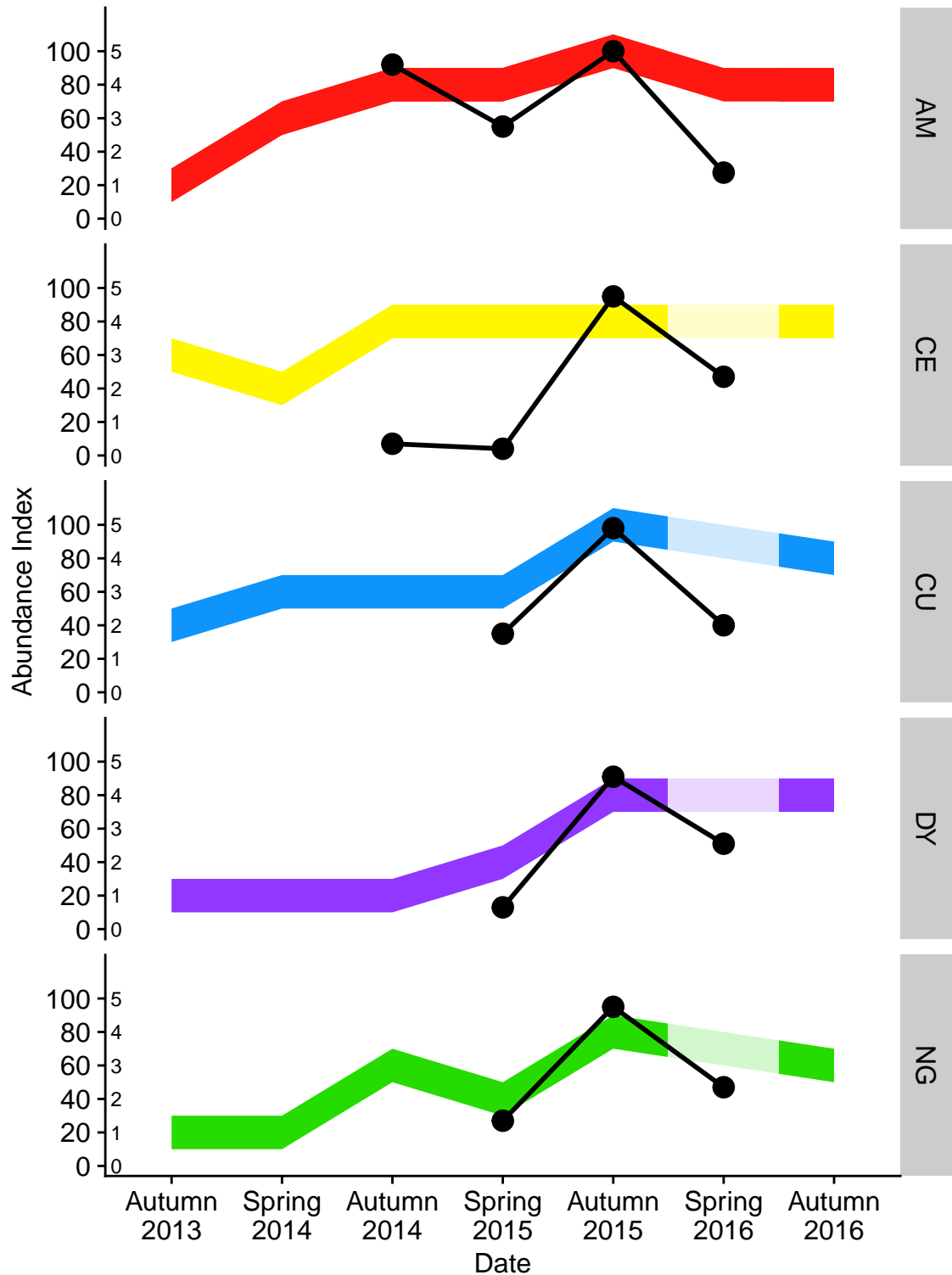
**Tab. 3.1.:** Summary of the total number of animals (N) and the number of males and females (M,F) collected during each sampling session at each sampling site, as well as the mean dry eye lens weight (in mg, standard deviation in parentheses), and the proportion of individuals reproductively active (males, females).

site	Date	N (M,F)	Lens Mass (mg)	% Reproductive (M,F)
AM	Autumn 2014	35 (11,24)		0.11 (0,0.17)
AM	Spring 2015	31 (15,16)	8.1 (2.6)	0.71 (0.8,0.62)
AM	Autumn 2015	42 (19,23)	6.9 (1.1)	0.45 (1,0)
AM	Spring 2016	30 (10,20)	7.3 (1.1)	0.83 (1,0.75)
CE	Autumn 2014	31 (12,19)		0.16 (0,0.26)
CE	Spring 2015	33 (22,11)	5.5 (1.8)	0.94 (1,0.82)
CE	Autumn 2015	33 (11,22)	5.9 (1.4)	0.33 (1,0)
CE	Spring 2016	31 (17,14)	7.0 (1.3)	0.87 (1,0.71)
CU	Spring 2015	34 (18,16)	6.8 (1.9)	0.85 (1,0.69)
CU	Autumn 2015	35 (8,27)	5.4 (1.7)	0.23 (1,0)
CU	Spring 2016	37 (16,21)	7.5 (2.1)	0.65 (0.69,0.62)
DY	Spring 2015	31 (16,15)	5.9 (1.9)	0.77 (0.94,0.6)
DY	Autumn 2015	30 (13,17)	4.8 (1.7)	0.47 (0.77,0.24)
DY	Spring 2016	32 (15,17)	6.5 (2.2)	0.69 (0.8,0.59)
NG	Spring 2015	34 (19,15)	7.1 (1.5)	0.97 (1,0.93)
NG	Autumn 2015	36 (16,20)	6.4 (1.4)	0.5 (1,0.1)
NG	Spring 2016	30 (16,14)	6.1 (2.6)	0.5 (0.44,0.57)

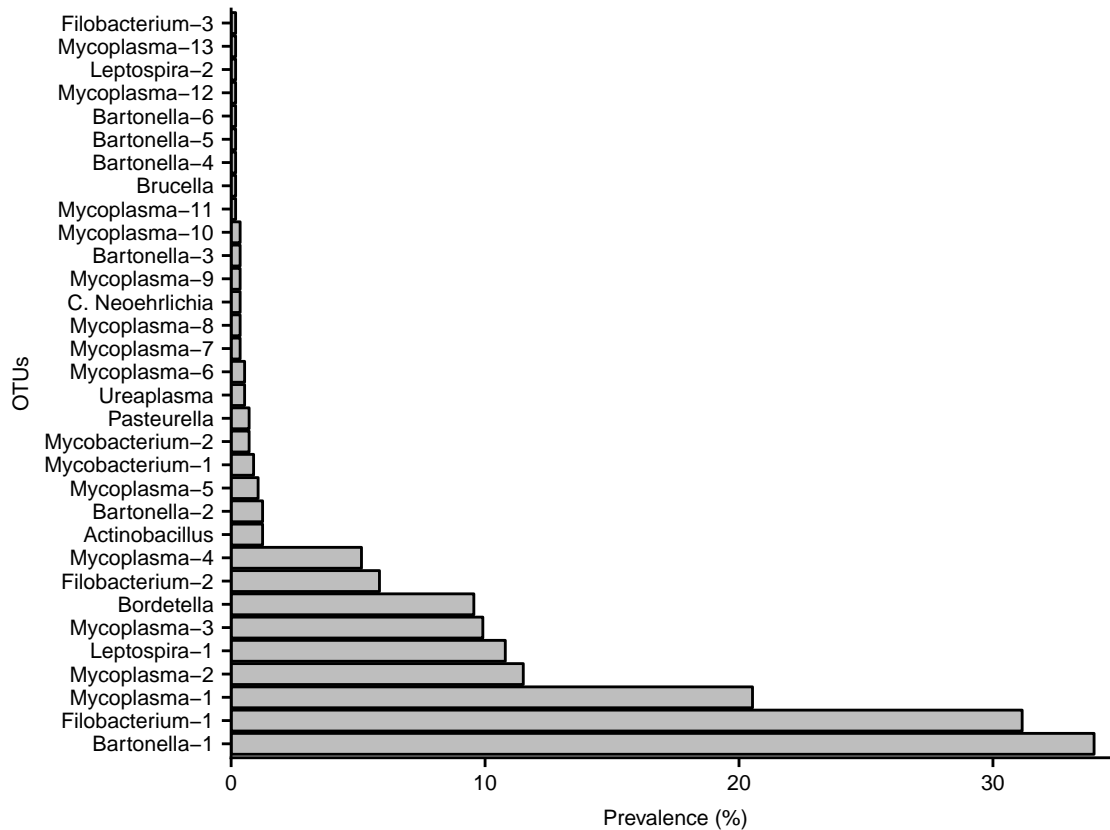
We obtained a total of 33,531,678 reads from the four runs pooled together, binned into 10,504 OTUs. After read filtering and the removal of OTUs identified as reagent-, environmental-, or gut-contaminants, 32 OTUs from 12 genera were labelled as parasites (Figure 3.2). Global prevalence ranged from 33 % (192 individuals) to 0.2 % (one individual), and genus richness ranged from 13 OTUs per genus (*Mycoplasma* sp.) to one OTU per genus.

### 3.3.2 Infracommunities

Infracommunity richness ranged from 0 to 9 OTUs/animal, with a mean of 1.49 OTUs/animal (SD = 1.47). When grouped by sampling session, mean infracommunity richness ranged from 0.61 OTUs/animal (SD = 0.74) to 2.48 OTUs/animal (Figure 3.4). Model selection to identify predictors of infracommunity richness resulted in 5 models



**Fig. 3.1.:** Abundance estimates for *A. terrestris* in five communes in Franche-Comté, France. Lines indicate changes in abundance indices estimated using walked line transects (longest transect, 1.2 km), while coloured bands indicate changes in abundance indices based on visual assessments conducted at the commune level. Pale sections of the bands in Censeau, Cuvier, Doye and Onglières indicate missing data (FREDON visual inspections of these communes were not conducted in spring of 2016).



**Fig. 3.2.:** Global prevalence of 32 OTUs identified as parasitic bacteria in *Arvicola terrestris* collected from five sampling sites in the region of Franche-Comté. Numbered suffixes are used to distinguish between OTUs that were assigned to the same genus; OTUs were formed by binning sequences together with a 97 % similarity cutoff.

for the full data set, and 8 models for the 2015-2016 data set (Table 3.2); in all but one model, the count component included study site and date. The zero component of the models of the complete data set all included current local abundance and reproductive status, while the zero component for the subset included age class in all but one model. Likelihood ratio tests indicated that further simplifying models (5) and (13) significantly reduced model fit, and likelihood ratio tests between the other candidate models that (5) and (13) were nested within indicate that additional terms do not significantly improve model fit.

Models (5) and (13) are summarized in Table 3.3; in both models, infracommunity richness is significantly lower in Censeau and Doye than in Arc-sous-Montenot, and infracommunity richness is significantly lower in spring than in autumn. Under model (5), zeros are best predicted by current local abundance (fewer zeros at higher abundance) but we failed to detect a significant relationship between zeros and reproductive activity. Under model (13), we failed to detect a significant relationship between zeros and age class.

**Tab. 3.2.:** Model selection results for infracommunity richness modelled (OTUs/animal) in *Arvicola terrestris* in Franche-Comté. Infracommunity richness was modelled using zero-inflated Poisson generalized linear regression with log link for the count component and a binomial distribution with logit link for the zero component. Terms used in model selection were current local (transect) abundance indices ( $t-ab_0$ ), site, sex, reproductive status, date (season and year) and commune-level abundance indices from the previous season (approx. 6 months prior,  $F-ab_{-6}$ ) and the previous year (12 months prior,  $F-ad_{-12}$ ).

	Data set	Count Component	Zero Component	df	deviance	AIC	$\Delta AIC$
(1)	2014	$t-ab_0 + \text{site} + \text{date}$	$t-ab_0 + \text{status}$	10	704.7	1749.87	1.85
(2)	-2016	$\text{site} + \text{sex} + \text{date}$	$t-ab_0 + \text{status}$	10	704.7	1749.86	1.84
(3)		$\text{site} + \text{date} + \text{status}$	$t-ab_0 + \text{status}$	10	704.8	1749.88	1.86
(4)		$\text{site} + \text{date}$	$t-ab_0 + \text{sex} + \text{status}$	10	704.9	1750.02	2.00
(5)		$\text{site} + \text{date}$	$t-ab_0 + \text{status}$	9	704.9	1748.02	0
(6)	2015	$\text{site} + \text{sex} + \text{date}$	age class	9	605.8	1507.92	2.00
(7)	-2016	$\text{site} + \text{date} + \text{status}$	age class	9	605.7	1507.84	1.91
(8)		$t-ab_0 + \text{site}$	age class	7	608.4	1507.53	1.60
(9)		$\text{site} + \text{date}$	$t-ab_0 + \text{age class}$	9	605.3	1507.45	1.53
(10)		$\text{site} + \text{date}$	$\text{sex} + \text{age class}$	9	605.6	1507.77	1.85
(11)		$\text{site} + \text{date}$	$\text{sex} + \text{status}$	8	607.7	1507.92	1.99
(12)		$\text{site} + \text{date}$	$\text{age class} + \text{status}$	9	605.2	1507.35	1.42
(13)		$\text{site} + \text{date}$	age class	8	605.7	1505.92	0

Infracommunity similarity, as measured by the Jaccard distance calculated between every pair of animals, exhibits a weak but statistically significant decline with distance as measured by Pearson correlations between Jaccard distance matrices and geographic distance matrices ( $r = 0.29$ ,  $p = 0.001$  in autumn 2014;  $r = 0.12$ ,  $p = 0.001$  in spring 2015;  $r = 0.12$ ,  $p = 0.002$  in autumn 2015;  $r = 0.09$ ,  $p = 0.004$  in spring 2016). PERMANOVA analyses with the complete data set and with just 2015-2016 data indicates that infracommunities are more similar within locations, dates, sexes,



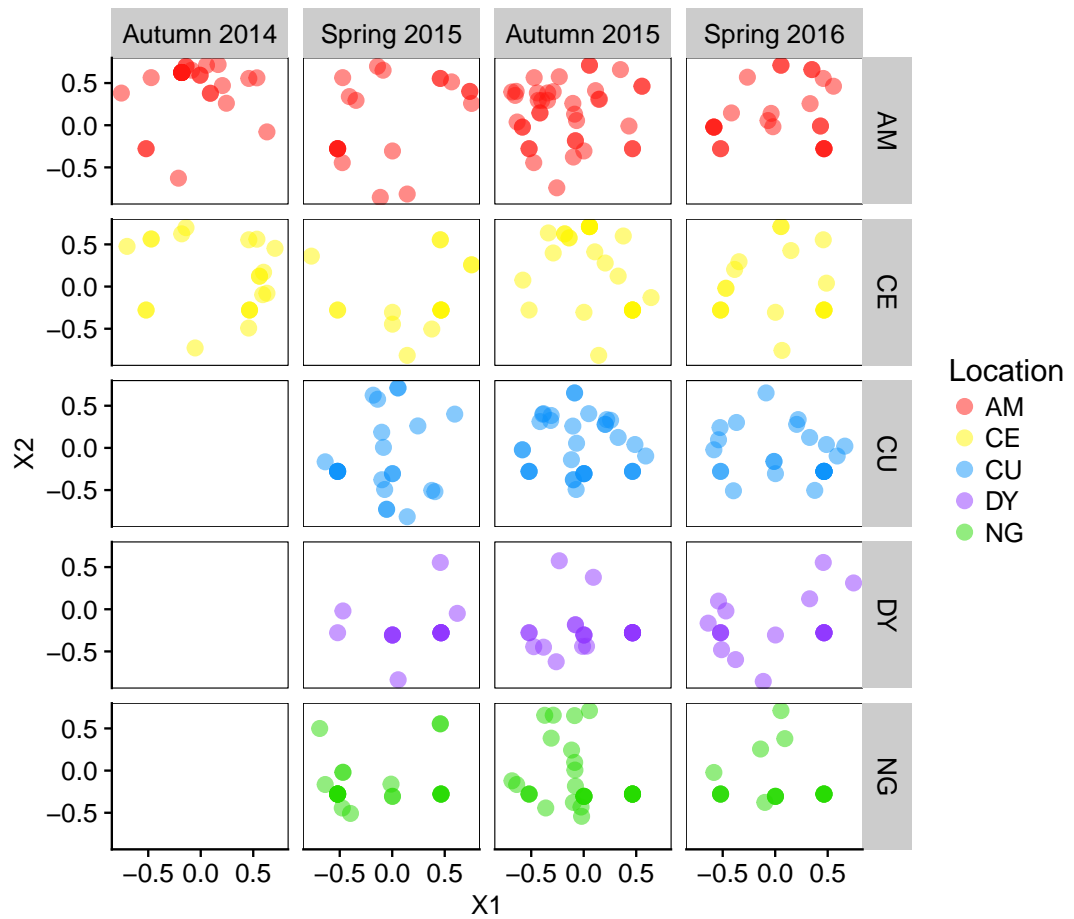
**Tab. 3.3.:** Parameter estimates for models (5) and (13) from Table 3.2.  $t-ab_0$  is current local abundance estimated by transect, and age classes were assigned based on dry lens weights (<5 mg - juvenile, 5 mg  $\leq$  and <7 mg - young adult, 7 mg  $\leq$  - older adult.

	Data set	Component	Term	Coefficient	standard error	z-value	p-value		
(5)	2014-2016	Count	(Intercept)	0.81	0.11	7.57	< 0.001		
			site - CE	-0.41	0.11	-3.75	< 0.001		
			site - CU	0.14	0.10	1.41	0.158		
			site - DY	-0.44	0.13	-3.49	< 0.001		
			site - NG	-0.17	0.11	-1.54	0.123		
			Spring 2015	-0.67	0.14	-4.87	< 0.001		
			Autumn 2015	0.10	0.12	0.84	0.403		
			Spring 2016	-0.44	0.13	-3.30	0.001		
			Zero	(Intercept)	-0.62	0.61	-1.02	0.308	
		$t-ab_0$	-3.46	1.56	-2.21	0.027			
		status	-12.61	287.34	-0.04	0.965			
		(13)	2015-2016	Count	(Intercept)	0.19	0.11	1.73	0.084
					site - CE	-0.40	0.12	-3.19	0.001
site - CU	0.14				0.11	1.36	0.174		
site - DY	-0.47				0.13	-3.66	< 0.001		
site - NG	-0.18				0.12	-1.59	0.111		
Autumn 2015	0.75				0.10	7.62	< 0.001		
Spring 2016	0.21				0.11	1.93	0.054		
Zero	(Intercept)				-8.42	29.39	-0.29	0.774	
young adult	-1.91				42.94	-0.04	0.965		
older adult	6.27			29.39	0.21	0.831			

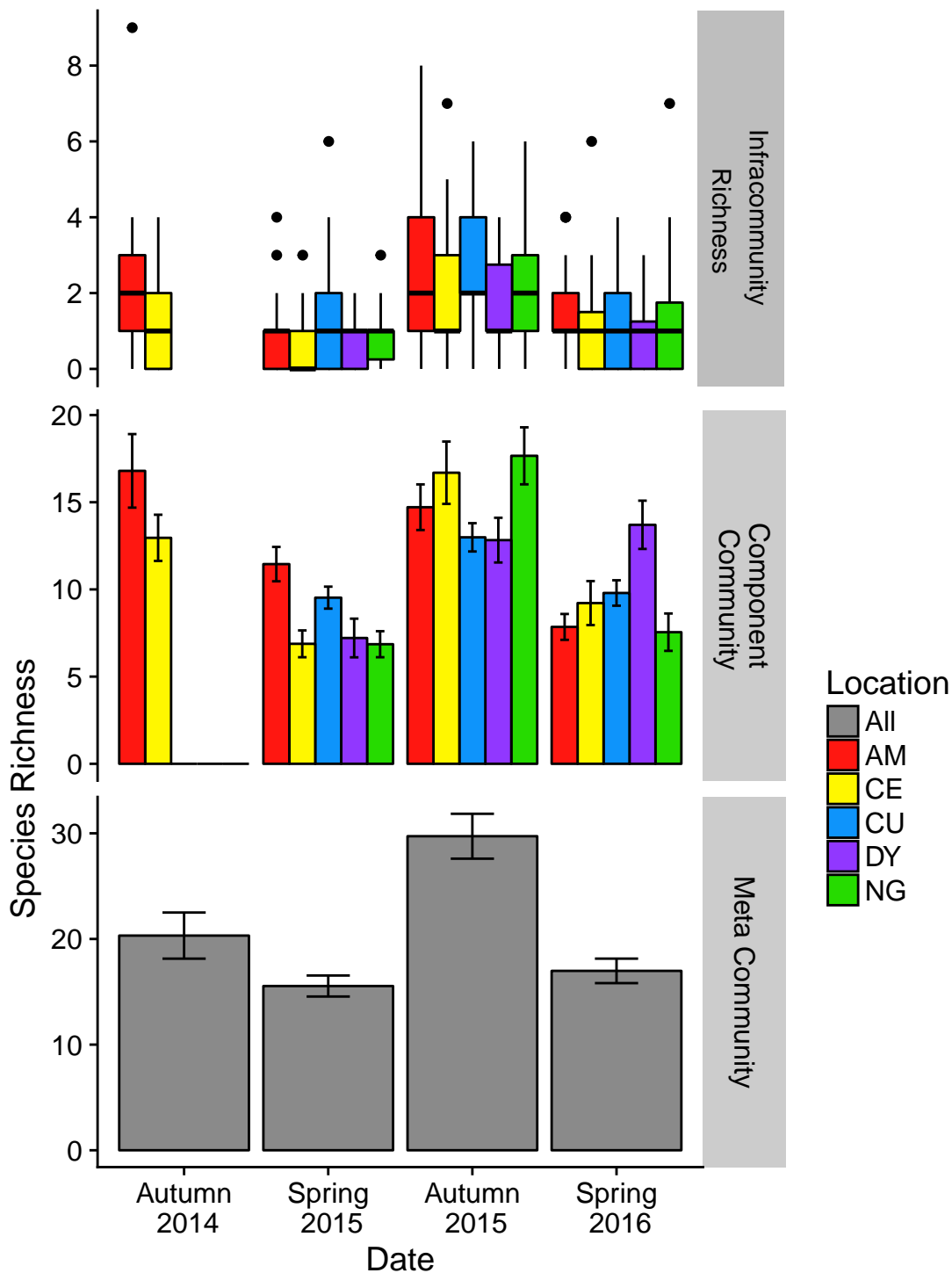
current abundances, and for animals in which lens weights were available, within age class (Table 3.4). However, inspection of the sums of squares for each term in the PERMANOVA indicates that statistically significant predictors do not account for much of the variability in the data; Figure 3.3, shows this graphically via nMDS ordination of Jaccard distances between infracommunities grouped by location and date. A lack of clear clustering within locations and dates supports the results of the PERMANOVA analysis in that there is minimal structure in infracommunity composition within the global data set.

### 3.3.3 Component Communities

Component community richness varied from 6.86 (standard error 0.75) OTUs/population to 17.7 (standard error 1.63) OTUs/population (Figure 3.4). Modelling selection results for the complete data set and data from 2015-2016 are shown in Table 3.5; likelihood ratio tests between the models with the lowest AIC values and the others in the table indicates that adding additional terms does not improve model fit, and removing terms also does not improve model fit. Using the full data set, component community richness is best predicted by current local abundance and date, but when using just data from 2015-2016, current local abundance alone is the best predictor of component community richness.



**Fig. 3.3.:** nMDS ordination of Jaccard distances between individual OTU communities (infracommunities). Each point represents a single animal's parasitic bacterial OTU community, points closer together in the ordination are more similar than those further apart.



**Fig. 3.4.:** a) Box plots (box and whisker diagrams) of bacterial infracommunity richness (individual parasitic bacterial OTU richness, OTUs/animal) in *Arvicola terrestris* collected from five locations in Franche-Comté from autumn 2014 to spring 2016. b) Bootstrapped component community richness (number of bacterial OTUs found in the population). c) Meta-community richness (number of OTUs found in all study sites for each sampling data). Error bars are standard error.

**Tab. 3.4.:** PERMANOVA results for infracommunity dissimilarities (Jaccard distance). Sums of squares are marginal (ie. type III).  $t\text{-ab}_0$  is current local abundance (transect-based),  $F\text{-ab}_{-6}$  and  $F\text{-ab}_{-12}$  are commune abundances from 6 months and a year prior and age classes (juvenile, young adult, older adult) were assigned based on dry lens weight. Factors were assessed for heteroskedasticity using the betadisper function of package vegan before being included in the models.

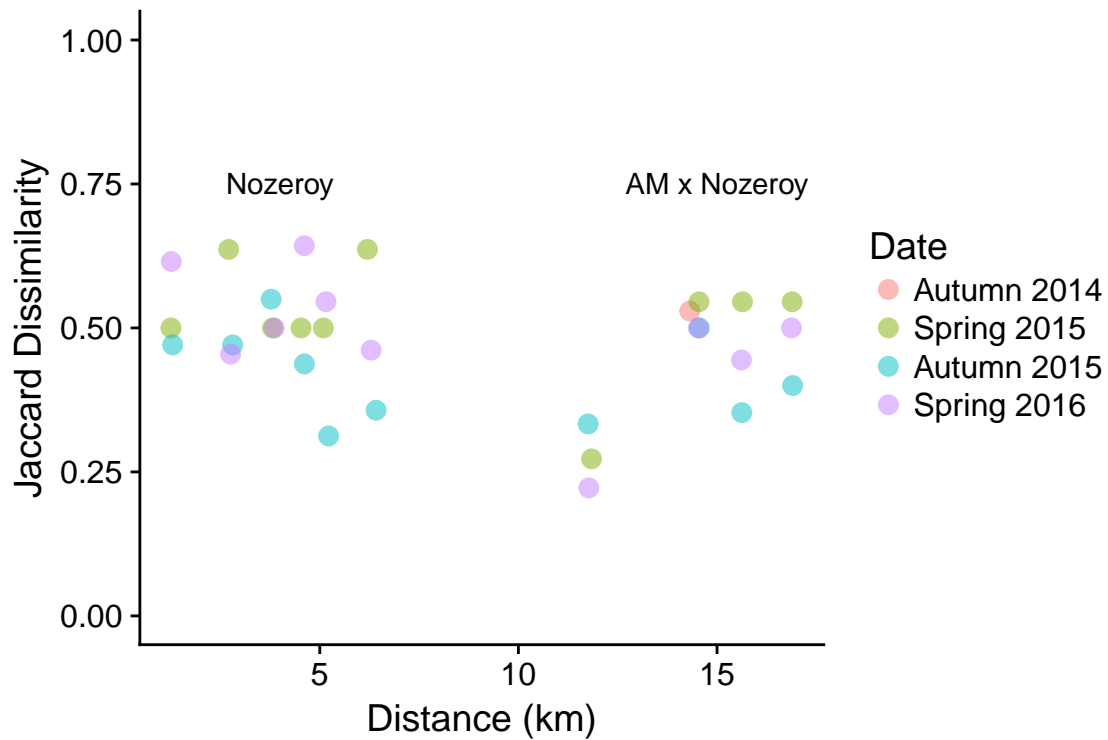
Data set	Term	df	Sums of Squares	F	p	heteroskedastic
2014 - 2016	site	4	3.85	3.09	0.001	yes
	date	3	4.13	4.43	0.001	no
	$t\text{-ab}_0$	1	2.44	7.85	0.001	no
	sex	1	1.26	4.06	0.001	no
	$F\text{-ab}_{-12}$	1	0.60	1.92	0.083	no
	$F\text{-ab}_{-6}$	1	0.28	0.90	0.455	yes
	Residual	398	123.81			
2015 - 2016	age class	2	3.45	5.74	0.001	yes
	site	4	3.02	2.51	0.001	yes
	date	2	2.34	3.89	0.001	no
	$t\text{-ab}_0$	1	1.08	3.60	0.007	no
	sex	1	1.34	4.47	0.001	no
	$F\text{-ab}_{-12}$	1	0.43	1.43	0.205	yes
	$F\text{-ab}_{-6}$	1	0.27	0.91	0.465	yes
	Residual	342	102.79			

Term coefficients and associated p-values for models (3) and (7) are shown in Table 3.6. Using the whole data set, component community richness increases with increasing density, and spring 2015 richness and spring 2016 richness is significantly lower than autumn 2014 richness. Using just data from 2015-2016, component community richness increases significantly with current local abundance.

**Tab. 3.5.:** Model selection result for component community richness modelled using a generalized linear model with Gaussian family and identity link. Parameters included were transect-based abundance ( $t\text{-ab}_0$ ), site, FREDON abundance from 6 months prior ( $F\text{-ab}_{-6}$ ) and 12 months prior ( $F\text{-ab}_{-12}$ ), date, and mean lens weight in 1/10 mg. An exhaustive search was performed; models were excluded if their maximum VIF score was higher than 10 and  $\Delta$  AIC score was less than 2.

	Data set	Model	df	deviance	AIC	$\Delta$ AIC
(1)	2014-2016	$t\text{-ab}_0 + F\text{-ab}_{-6} + \text{date}$	5	43.8	78.34	1.98
(2)		$t\text{-ab}_0 + F\text{-ab}_{-12} + \text{date}$	5	40.2	76.88	0.51
(3)		$t\text{-ab}_0 + \text{date}$	4	43.9	76.36	0
(4)	2015-2016	$t\text{-ab}_0 + F\text{-ab}_{-6}$	2	40.2	65.37	2.00
(5)		$t\text{-ab}_0 + F\text{-ab}_{-12}$	2	39.2	64.98	1.61
(6)		$t\text{-ab}_0 + \text{mean lens weight}$	2	39.4	65.07	1.70
(7)		$t\text{-ab}_0$	1	40.2	63.37	0

Mantel tests indicate that Jaccard distance does not increase with increasing geographic distance between populations ( $r = 0.19$ ,  $p = 0.23$  spring 2015;  $r = -0.41$ ,  $p = 0.85$  autumn 2015;  $r = -0.40$ ,  $p = 0.83$  spring 2016). This is shown graphically in Figure 3.5.



**Fig. 3.5.:** Jaccard distances plotted against geographic straight-line distances between sampling site trap centroids for each sampling period (i.e. Jaccard and geographic distances between sampling sessions of different dates are not shown). Geographic distances < 10 km are between Censeau, Cuvier, Onglières and Doye, which are all found in the Nozeroy zone of continuous grassland. Distances between these four sites and Arc-sous-Montenot (AM) are annotated as AM x Nozeroy

**Tab. 3.6.:** Parameter estimates for models (3) for and (7) from Table 3.5. t-ab<sub>0</sub> is current local abundance (transect-based)

Model	Data set	Term	Coefficient	standard error	t-value	p-value
(3)	2014-2016	Intercept	11.68	1.85	6.30	< 0.001
		t-ab <sub>0</sub>	6.46	2.56	2.52	0.027
		Spring 2015	-5.03	1.70	-2.95	0.012
		Autumn 2015	-2.89	1.99	-1.45	0.172
		Spring 2016	-4.80	1.61	-2.98	0.011
(7)	2015-2016	Intercept	5.64	0.91	6.20	< 0.001
		t-ab <sub>0</sub>	9.72	1.43	6.78	< 0.001

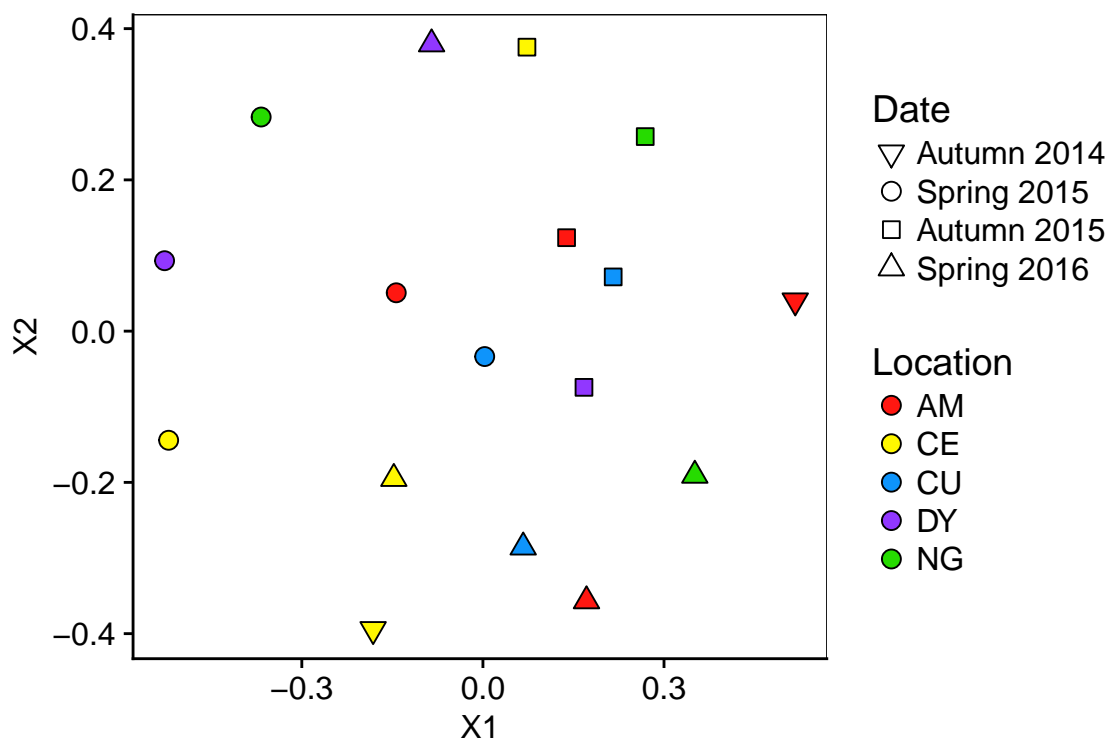
**Tab. 3.7.:** PERMANOVA results for component community Jaccard distances. t-ab<sub>0</sub> is transect-based current local abundance, F-ab<sub>-6</sub> is commune-level abundance from 6 months prior, and F-ab<sub>-12</sub> is commune-level abundance from 12 months prior.

Data set	Term	df	Sums of Squares	F	p	heteroskedastic
2014 - 2016	site	4.0	0.53	1.42	0.099	no
	date	3.0	0.53	1.88	0.010	no
	t-ab <sub>0</sub>	1.0	0.16	1.70	0.092	
	F-ab <sub>-6</sub>	1.0	0.21	2.20	0.031	yes
	F-ab <sub>-12</sub>	1.0	0.11	1.18	0.291	no
	Residual	6.0	0.56			
2015 - 2016	site	4.0	0.55	1.69	0.056	no
	date	2.0	0.36	2.18	0.034	no
	t-ab <sub>0</sub>	1.0	0.24	2.97	0.009	
	F-ab <sub>-6</sub>	1.0	0.24	2.91	0.017	yes
	F-ab <sub>-12</sub>	1.0	0.11	1.38	0.238	no
	mean lens weight	1.0	0.09	1.13	0.345	
	Residual	4.0	0.33			

PERMANOVA analysis of component communities of the complete data set indicates that communities sampled at the same date and with the same commune-level abundance from the previous season were more similar; for the reduced data set, current transect-based abundance was also a significant predictor of similarity. nMDS ordination of Jaccard distances between component communities are shown in Figure 3.6.

### 3.4 Discussion

We present here the first results of an investigation into bacterial parasites in *Arvicola terrestris* populations in the Franche-Comté region of France. The 32 bacterial OTUs that we have included in our analysis here represent 11 genera, some of which have been detected in *Arvicola* sp. or other wild rodent populations in Europe; Gelling et al. (2012) reported *Leptospira* sp. and *Bartonella* sp. in Scottish *Arvicola amphibus*, and *Candidatus* Neoehrlichia has been reported in French bank voles (*Myodes glareolus*) (Vayssier-Taussat et al., 2012) and *Mycoplasma coccoides* has been detected in Swiss populations of wood mice (*Apodemus sylvaticus*) and *M. glareolus* (Willi et al., 2007). In addition to the wide taxonomic diversity we detected, the bacterial parasites also vary



**Fig. 3.6.:** nMDS ordination of Jaccard dissimilarities of bacterial parasites component communities of Arc-sous-Montenot (AM), Censeau (CE), Cuvier (CU), Doye (DY), and Onglières (NG).

widely in their transmission dynamics, the tissues they tend to parasitize, their effects on hosts, and their general ecology. We provide more detailed overviews of all of the genera we have detected in the conclusion and appendices of this thesis, and limit ourselves to a few comments here.

Transmission routes of the bacteria we detected vary, and include direct contact (e.g. *Bordetella*; Register and Harvill, 2010), aerosols (e.g. some mycoplasmas; Whithear, 2001), bodily fluids like urine (e.g. *Leptospira*; Adler and de la Peña Moctezuma, 2010) and vectors (e.g. *Bartonella* and *Candidatus Neohelminthosiphium*; Brenner et al., 2005b; Silaghi et al., 2012). Localization within the host also varies, with some bacteria targeting red blood cells (e.g. *Bartonella* and hemotropic mycoplasmas, Brenner et al., 2005b; Messick, 2004), the kidneys (*Leptospira* Adler, 2015), the respiratory tract (e.g. some mycoplasmas, Brown et al., 2001), and given its role in immune function and clearing bacterial infections the spleen is also often host to parasitic bacteria (Deng et al., 2012). Regarding diseases, some of these bacteria are serious problems in livestock but in wild populations and specifically rodents, little is known about their potential to cause mortality or alter other aspects of host biology like reproductive rate, dispersal or risk of predation.

Model selection indicates that sampling site and date are significant predictors of infracommunity richness, with site and date appearing in the count component of all but one model retained during model selection; intrinsic host characteristics like sex and reproductive status appeared in the count components of some models but likelihood ratio tests indicate that including these terms did not result in a better-fit model. This was found to be the case for both the complete data set and just data from 2015-2016. Similar patterns of extrinsic factors, rather than intrinsic host characteristics, being more strongly correlated with infracommunity richness have been reported in other rodent-parasite systems, although direct comparisons to other studies are difficult due to differences in how ages are classed and whether host abundance is also considered: infracommunity richness in haemoparasites (*Babesia*, *Bartonella*, *Haemobartonella* (*Mycoplasma*), *Hepatozoon*, *Trypanosoma*) in *Myodes glareolus* in Poland was significantly correlated with study site but not host sex or age (Bajer et al., 2014), and Pawelczyk et al. (2004) reported strong seasonal and annual differences in infracommunity richness of the same haemoparasites in a population of Polish *Microtus arvalis*, with some evidence that age was important as part of an interaction with year. These two studies assigned age class based on body weight and reproductive status, and neither of these studies considered host abundance or density as a potential explanatory variable for infracommunity richness.

Zero components of the "best" models for the full data set and the 2015-2016 data exhibit the opposite trend; host intrinsic factors like current local abundance and reproductive status (the full data set) and age class (the 2015-2016 subset) were included in the models retained after model selection, and extrinsic factors like date and site are not included in the zero component of any model. Neither status in the full data-set model nor age class in the 2015-2016 model were statistically significant, leaving just local current abundance in the full data set as a significant predictor of infection by one or more bacterial parasites.

That host intrinsic factors are in general not significant predictors of infracommunity richness may be related to the transient nature of some bacterial infections, as well as interspecific interactions; for example, *Bartonella* infections can be as short as a month (Birtles et al., 2001), and some interspecific interactions serve to lengthen or shorten infection duration (Telfer et al., 2008). Cross-sectional "snap-shot" sampling, as we have done here, doesn't allow us to assess the impact such interactions might have on infracommunity richness at any given time, and also leaves us vulnerable to stochasticity masking real differences in infection probabilities across sexes or age classes.

Significant differences in infracommunity richness between sites was somewhat surprising given their close proximity. The five sites are generally similar in that they are all grassland parcels, but there are some differences in local landscape composition; Arc-sous-Montenot, Cuvier, and Onglières are surrounded on all sides by more uncultivated



tivated grassland, while Censeau and Doye trapping sites are adjacent to parcels that were cultivated with cereal over the period that we sampled. Arc-sous-Montenot, Cuvier and Onglières are also closer to forest habitat than Doye and Censeau, which are more centrally located in the Nozeroy grassland zone. Landscape composition wasn't found to be an important predictor of *Trichuris arvicolae* infection in *A. terrestris* in this region, but landscape composition at the scale of 1 km was found to be a significant predictor of cowpox antibodies in *A. terrestris* in this region (Charbonnel et al., 2008a).

Infracommunity richness does not appear to vary between sexes, which is likely related to *A. terrestris* living habits; males and females cohabit tunnel systems (Airoldi, 1976a). This result is consistent with reports from some other systems, including haemoparasites in spiny mice *Acomys dimidiatus* in Egypt (Alsarraf et al., 2016) and the same haemoparasites in Polish *Microtus arvalis* and *Myodes glareolus* (Pawelczyk et al., 2004; Bajer et al., 2014). Similar results were found in regards to helminth parasitism in *A. terrestris* in our study region; Cerqueira et al. (2007) reported that helminth infracommunity richness in voles collected in 1995-1996 did not vary between sexes.

We failed to find evidence of age as a significant predictor of infracommunity richness; this is consistent with the results reported by Bajer et al. (2014), who found that while prevalence of some haemoparasites (namely *Bartonella*, *Haemobartonella* and *Trypanosoma*) in *Myodes glareolus* varied with age class, infracommunity richness didn't. In *A. terrestris* collected from Cuvier, Doye, and Onglières (and neighbouring communes) in 2003, the presence of cowpox antibodies (indicating past infection with cowpox virus) did not differ with age, although in this case, age was estimated directly from lens weights rather than binned into classes, and the authors were tentative in this conclusion given that low numbers of juveniles were captured during this study (Charbonnel et al., 2008a). Conversely, infection by *Taenia taeniaformis* in Franche-Comté *A. terrestris* and *Taenia taeniaformis* and *Echinococcus multilocularis* in *A. terrestris* in neighbouring Switzerland was found to be biased towards older individuals (Burlet et al., 2011; Deter et al., 2006). This discrepancy may be due to the duration of infection of these two groups of parasites; in *Apodemus sylvaticus* and *Myodes glareolus*, infections by the haemoparasite *Bartonella* sp. can be cleared by the immune system relatively quickly (within a month), and infection doesn't appear to convey immunity against subsequent infections (Birtles et al., 2001). Infection by *Taenia taeniaformis* and *Echinococcus multilocularis* on the other hand is lifelong and *Taenia taeniaformis* doesn't appear to cause additional mortality in *A. terrestris*, thus infections can accumulate in the population as cohorts age (Deter et al., 2006).

The use of age classes rather than age estimates may have obscured some real age-related patterns in infracommunity richness; several formulae have been developed to convert lens weight to age in months for *A. terrestris* (e.g. Morel, 1981) but these have been based on small sample sizes and were found to differ between populations of *A.*

*terrestris*. In addition, lens weights can be affected by sex (Jánová et al., 2007), body condition and reproductive status (Jánová et al., 2007), and season (Martinet and Spitz, 1971), thus applying a conversion formula developed using other populations without corrections for sex, season, and reproduction is likely to produce biased age estimates.

We detected a statistically significant increase in infracommunity distances with increasing geographical distances between hosts for all four dates, but this correlation wasn't very high, especially after autumn 2014 when Spearman rank correlations between Jaccard and geographic distances dropped below 0.15. Jaccard distances between infracommunities from the same sampling session are fairly high to begin with (mean distance of 0.72, standard deviation = 0.3), thus Jaccard distance may quickly saturate with geographic distance. Correlations between just Arc-sous-Montenot and Censeau in later dates are similarly low ( $r = 0.18$ ,  $p = 0.002$ ,  $r = 0.17$ ,  $p = 0.001$ ,  $r = 0.13$ ,  $p = 0.001$  for spring 2015, autumn 2015, and spring 2016.), so the lower correlations in 2015-2016 are not due to the inclusion of the other sites in the analysis.

At the component community scale, geographic and Jaccard distances were not significantly correlated for any date, even when distances were partitioned into within-Nozeroy and Arc-sous-Montenot x Nozeroy groups. This may be related to the scale at which our sites were separated; though *A. terrestris* dispersal distances in Franche-Comté are rarely over 100 m (Saucy, 1988), Berthier et al. (2005) found that genetically homogeneous populations in Franche-Comté could be found at scales up to 20 km, and Telfer et al. (2003b) reported dispersal distances as large as 5.2 km in Scottish wetland populations, which suggests that our study sites, especially the four in Nozeroy, are likely not very isolated.

PERMANOVA results indicate that the other factors we tested don't account for much of the variation in infracommunities; if we consider the proportions of explained variability (i.e. redundancy as defined by Gittins (1985), the proportion of multivariate variability explained by factors), the statistically significant factors do not account for much; site, date, current local abundance and sex together account for just 7 % of the multivariate variability, and given that site was found to be significantly heteroskedastic, the inclusion of this factor needs to be approached with caution. Inspection of Figure 3.3 supports this general result that there is a lot of variability between infracommunities within sites and dates; there is little evidence of clustering through time within sites, or between sites sampled at the same time.

Conversely, PERMANOVAs indicate that 25 % of variation in component communities can be accounted for by date alone - sampling sessions from the same date are more similar to each other than to other dates. That local current abundance is also a significant factor in 2015-2016 data may be related to a correlation between abundance and date,

and additional data from the low-abundance period should prove useful in disentangling abundance and seasonal affects.

At component community scale, model selection shows that local abundance is an important predictor of richness as it appears in every model retained after model selection for both the complete data set and just data from 2015-2016. Date was found to be significant in the full data set but not in the data from 2015-2016, likely because aside from Censeau in autumn 2014, local current abundance is strongly correlated with date. That abundance is important at this scale conforms to predictions made using the island biogeography framework applied to host-parasite systems - smaller populations are more likely to fall below the minimum abundance required to sustain a given parasite's presence in the population (Swinton et al., 2002), leading to lower component community richness compared to larger populations.

Meta community richness also changes through time, which suggests that component community richness isn't just increasing because of exchange between the populations we studied - other sources of bacteria are likely contributing to component communities. Alternative sources of bacteria include other populations of *A. terrestris*; Censeau, Cuvier, Doye and Onglières are located in the northern corner of the Nozeroy zone, which is approx. 60 km<sup>2</sup> of pasture, meadows and some cereal cultivation bounded by forest. Previous investigations into the genetic structure of the *A. terrestris* populations in this zone suggest that at high abundance they are homogenized by immigration (Berthier et al., 2006), thus our sampling locations are likely to be subject to genetic and perhaps parasite exchanges not only between each other but also from other populations within the zone. This could explain the increase in meta community richness in autumn 2014 and 2015, as well as increases in component community richness without increases in similarity between those component communities; "new" parasites may be introduced from other populations that were not sampled.

Further directions for research should include an exploration of patterns of co-occurrence in the OTUs we have reported here; interactions between parasites have important epidemiological implications and may have contributed to some of the patterns we have observed here. For example, *Bartonella taylorii* infections in *Microtus agrestis* are longer if the animal has already been infected with cowpox, and are shorter if the animal has already been infected with *Babesia microti* (Telfer et al., 2008). Cowpox is present in *A. terrestris* in Franche-Comté (Charbonnel et al., 2008a), and may have influenced the prevalence of *Bartonella* sp. and other parasites here.

In addition, not all recorded variables were available for all sampling sessions; transect based abundances for previous years were not available for the first sampling session of each location, and current FREDON abundance indices were not available for all sampling sessions either. Therefore, current transect-based abundances were used and

FREDON abundances from the previous season and previous year were used to test questions regarding the effects of current and past abundance on variables of interest.

Several of the variables that we included in our linear modelling and PERMANOVA analyses were collinear; for example, at the population level, abundance and the proportion of the population that was reproductively active was highly correlated (Pearson's  $r = -0.87$ ), which is likely due to collinearity between transect-based abundance and season (point-biserial correlation coefficient  $r_{pb} = -0.9$ ). Higher abundances were restricted to autumn sampling sessions, which were conducted in late autumn after the main reproductive period had ended (Delattre and Giraudoux, 2009). Excluding models with high VIF scores during model selection ensured that we didn't include highly collinear terms in the same model, but we are left with the problem that while one factor may be a better predictor of infracommunity or component community richness, we haven't eliminated the possibility that other collinear factors are important.

The question now remains: are these same patterns preserved over the course of the decline phase? Charbonnel et al. (2008b) observed marked changes in body condition and immunocompetence of *A. terrestris* individuals over the course of a decline in Cuvier, Doye, and Onglières in 2003-2005, which could have serious implications for patterns of bacterial parasite infections over the course of this iteration of the decline.

## Summary

- *A. terrestris* populations were sampled in five locations in spring and autumn from 2014-2016
- infracommunity richness (the number of bacterial parasite OTUs detected within an individual host) is correlated with sampling location and date, with animals collected in autumn having higher richness than animals collected in spring
- component community richness (the number of bacterial parasite OTUs detected within a host population) is positively correlated with local abundance
- Infracommunity composition is highly variable, even between animals from the same population
- Component community composition appears to be correlated with date, with populations sampled at the same time more similar to each other than to earlier or later samplings from the same location
- geographic isolation is not a strong predictor of community composition similarity at the infr- or component community scale

# Long-Term Changes in *A. terrestris* Bacterial Parasite Communities

## 4.1 Introduction

In the previous chapter of this thesis we explored spatial variation in parasite communities over a relatively short time frame: the high-abundance phase from 2014-2016. Past investigations into *A. terrestris* populations in this region have been similarly short, leading to detailed information about the phase-related and spatial variability in helminth communities (Cerqueira et al., 2007), stress (Charbonnel et al., 2008b), age structure (Cerqueira et al., 2006), reproduction (Charbonnel et al., 2008b), and single-parasite prevalences (Charbonnel et al., 2008a; Deter et al., 2007; Deter et al., 2006). But there is little information about how these patterns might vary from one outbreak to the next, which is crucial if we wish to understand the processes underlying these patterns.

In addition, as we described in chapter 1 of this thesis, outbreaks can cause serious economic damage (Delattre and Giraudoux, 2009; Schouwey et al., 2014) and increase health risk to humans (Viel et al., 1999), and while the current management tool kit can be effective in reducing the likelihood of outbreaks and the damage caused by outbreaks locally (Schouwey et al., 2014; Couval and Truchetet, 2014), there remains an interest in predicting when the decline will occur. Pascal (1988) suggested that the reproductive status of females could act as indicators of the decline, as the majority of females they captured during the decline had placental scars while females caught the previous year did not. They also suggested that the number of dispersing individuals relative to non-dispersers was lower during the decline than in the previous year; however, the method used to distinguish between dispersers and sedentary individuals was somewhat arbitrary. Like the studies listed above, these potential indicators have not been further explored during later outbreaks, and from an effort perspective neither is ideal; relying on only one sex is wasteful as current trapping methods do not target one sex, and the method used to estimate the proportion of dispersers involved labour-intensive live trapping removals.

Thus, our ecological goals intersect with a management application: we are interested in how bacterial parasite communities change between outbreaks, and bacterial parasites may also be useful in predicting the decline phase of the cycle. We use *A. terrestris*

spleens collected from the cantons of Cuvier, Doye and Onglières in spring and autumn of 2003-2005 and 2015-2016 to address these questions; we use only spleens because other organs like the heart and kidneys were not all retained from the animals captured in 2003-2005. We have discussed in Chapter 2 that restricting our analysis to spleens alone is likely to underestimate the bacterial parasite community richness within vole individuals, and it is likely that we will also underestimate the prevalence of the individual bacterial species. We address this limitation by using an indicator approach: an indicator simplifies and summarizes complex information in such a way as to facilitate communication, and is used with the assumption that doing so is an accurate cost-effective alternative to collecting information on the many ecological processes, properties, or phenomena that it summarizes (National Research Council, 2000).

We use the Dufrene-Legendre group-corrected *IndVal* metric to identify indicator species of the late high-abundance phase within the splenic bacterial communities of the three communes; this metric has been used to identify indicator taxa in systems ranging from deep-sea vents (e.g. Cuvelier et al., 2017) to human gut communities (e.g. Bäckhed et al., 2015), and because it is based upon the relative abundance of a given taxon, as opposed to the absolute abundance, it can be particularly useful when using taxa with very different abundances within the data set, or taxa whose abundance changes drastically with time (McGeoch and Chown, 1998).

We asked the following questions: first, do we find the same bacterial OTUs in spleens from 2003-2005 and 2015-2016? Population abundance at the three sites has fluctuated in the intervening period between 2005 and 2013, and at low host abundances parasite species are expected to be more vulnerable to extinction events (Poulin and Morand, 2004), which may lead to changes in the parasite community composition over the long term. Second, are there single bacterial OTUs that are indicators of the late high-abundance phase of the 2003-2005 population cycle of *A. terrestris* in Doye, Onglières and Censeau? Is there a "best" scale or resolution for identifying potential indicators? If indicator species are identified in 2003-2005 samples, are they present in the *A. terrestris* populations in Doye, Onglières and Censeau from 2015-2016, and if so, do they exhibit similar patterns of prevalence as observed within the populations sampled in 2003-2005?

## 4.2 Materials and Methods

Study sites, animal trapping, dissection, and molecular methods are described in Chapter 2 of this thesis, and briefly recounted here. The communes of Cuvier, Doye and Onglières were visited in spring and autumn of 2003-2005 and 2015-2016; trapping locations differed slightly between the 2003-2005 and 2015-2016 sampling sessions (Table 2.1,23)

due to land-use changes in the intervening years - in Onglières, the original sampling site is no longer grassland and is instead scrub, and therefore not favourable habitat for *A. terrestris*. Animals captured in 2003-2005 were taken to the lab and housed for 4 days for an immunological experiment detailed in Charbonnel et al. (2008b) before being euthanized by cervical dislocation and dissected. Spleens were stored in RNAlater and frozen at -80C. Animals trapped in 2015-2016 were euthanized and dissected on site; spleens were stored in RNAlater and frozen at -20C.

DNA was extracted from spleens and the 16S rRNA gene was amplified via PCR using indexed primers to identify amplicons from individual animals (each DNA sample was amplified in duplicate). Amplicons were sequenced using the MiSeq Illumina platform, and the resulting sequences were filtered for quality and binned into operational taxonomic units (OTUs) with a 97 % similarity cutoff using the program mothur and the SOP developed by Patrick Schloss (Kozich et al., 2013). Sequences were classified using the Silva SSU database as a reference library, and false positives were removed if an OTU was found in only one of the two PCR replicates for each sample. OTUs were filtered to remove reagent contaminants (identified using negative and positive controls included in the DNA extraction and PCR steps), as well as environmental and gut bacteria (see Chapter 2 Box 2.2: Gut bacteria and environmental bacteria as opportunistic pathogens in animals).

## 4.2.1 Statistical Analyses

All statistics were performed using the software R (version 3.4.0, Team, 2012). Because we don't have data from autumn 2016 or later (the decline and low-abundance phases of this outbreak) we restrict our assessment of temporal variability in *A. terrestris* bacterial parasite communities to a simple presence/absence comparison of the OTUs identified in the 2003-2005 spleens and the 2015-2016 spleens.

To identify potential indicators of the late high-abundance phase, we began by assigning 2003-2005 sampling sessions to cycle phases based on vole abundance patterns through time (Figure 4.3). 2005 is clearly a low-phase year for all three locations; autumn vole abundances were all less than 5 %. Autumn vole abundances in 2004 in all three locations were considerably higher (above 60%) and are therefore classified as late high-abundance. 2003 vole abundances, all high (100%), were classified as early high-abundance.

We used the group-equalized Dufrene-Legendre Indicator value ( $IndVal_{ind}^g$ ), to identify indicator species for the late high-abundance phases of the 2003-2005 cycle. Using the terminology employed by De Cáceres and Legendre (2009), the group-equalized  $IndVal_{ind}^g$  for a given taxon in a given group of sites is the product of  $A_{ind}^g$  and  $B_{pa}$ ,



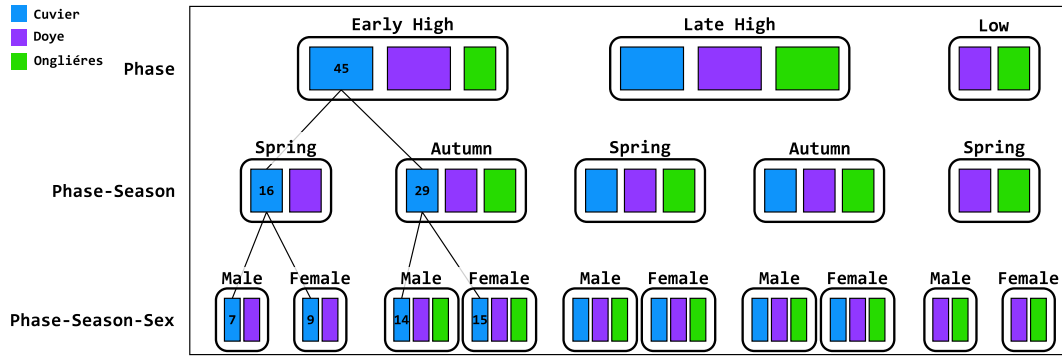
**Tab. 4.1.:** Example of how the  $IndVal_{ind,max}^g$  is calculated for each OTU at the phase-season group level. Prevalences for Filobacterium - 1 (Filo. - 1) and Mycoplasma - 4 (Myco. - 4) are shown for each sampling session, grouped by phase and season.  $A_{ind}^g$  for each group is calculated as the mean prevalence of the OTU in that group, divided by the sum of the mean prevalences of all groups.  $B_{pa}$  is calculated for each group as the number of sites within the group positive for the OTU, divided by the total number of sites within the group.  $IndVal_{ind}^g$  is the product of  $A_{ind}^g$  and  $B_{pa}$ , and  $IndVal_{ind,max}^g$  (shown in bold for the two OTUs) is the maximum  $IndVal_{ind}^g$ . P-values were estimated using the permutation method described by Dufrene and Legendre (1997) (1000 iterations).

	early high					late high					low		
	spring		autumn			spring			autumn		spring		
	CU	DY	CU	DY	NG	CU	DY	NG	CU	DY	NG	DY	NG
Filo. - 1	0	0	0	0	0	3	0	0	11	10	19	0	0
$A_{ind}^g$	0		0			0.08			0.92		0		
$B_{pa}$	0		0			0.33			1		0		
$IndVal_{ind}^g$	0		0			0.03			<b>0.92</b>		0		
p-value	1		1			0.6			<b>0.001</b>		1		
Myco. - 4	31	0	27	42	5	0	6	11	11	14	13	7	5
$A_{ind}^g$	0.24		0.38			0.08			0.19		0.09		
$B_{pa}$	0.5		1			0.67			1		1		
$IndVal_{ind}^g$	0.12		<b>0.38</b>			0.06			0.19		0.09		
p-value	0.63		<b>0.05</b>			0.89			0.39		0.73		

where  $A_{ind}^g$  is the mean abundance of the taxon within the target group divided by the mean abundance of the taxon over all groups in the data set, and  $B_{pa}$  is the relative frequency of the taxon within the target group (i.e. the number of sites in the group in which the taxon is found, divided by the number of sites within the group).  $IndVal_{ind,max}^g$  for a given taxon then is the maximum  $IndVal_{ind}^g$  when  $IndVal_{ind}^g$  is calculated for all groups in the data set. See Table 4.1 for a worked example.

$A_{ind}^g$  can be interpreted as the positive predictive power of the taxon, or the probability that a site belongs to the target group of sites given that an individual of the taxon is found;  $B_{pa}$  is the sensitivity of the taxon, or the probability of finding the taxon within a site when that site belongs to the group of interest (De Cáceres and Legendre, 2009).

We calculated  $IndVal_{ind,max}^g$  for OTUs found in the 2003-2005 spleens using several different group definitions in order to determine if sex and season could also be used to identify an indicator. We include sex because it can be identified in the field (although as mentioned in the introduction, indicators that rely on one sex can be wasteful), and season because of the effort associated with trapping multiple times a year - a sufficiently powerful and sensitive indicator that relies on trapping in only one season is preferable to a similarly powerful and sensitive indicator that relies on data from multiple trapping sessions. The narrowest definition used season, sex and phase to sort sampling sessions into groups, the second definition used phase and season, and the coarsest definition

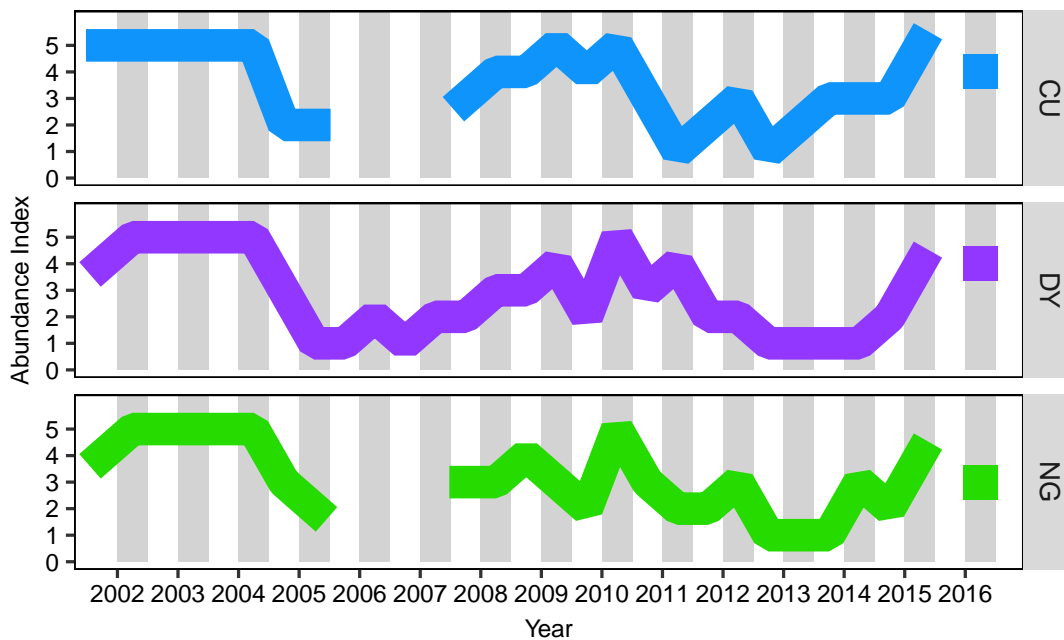


**Fig. 4.1.:** Illustration of the scales used to determine if season or sex are important. Rectangles represent the number of animals in each site after pooling at each scale.

used just phase. We used the *indicators* function from the *indicpecies* package with the *indval.g* function and a *max.order* of 1. Due to differences in the number of animals taken from each location during each sampling session, we have used taxon prevalence instead of taxon abundance.

$IndVal_{ind,max}^g$  p-values were estimated using the permutation method with 1000 iterations (Dufrene and Legendre, 1997). Significant  $IndVal_{ind,max}^g$  were then compared across the different scales with the objective of identifying high  $IndVal_{ind,max}^g$  at moderate resolution; too broad (i.e. phase) would require sampling in both spring and autumn, while too focused (one sex in one season in one phase) would be wasteful as typical live-trapping methods do not discriminate between the sexes, and captured individuals of the "wrong" sex would not be informative.

While the Dufrêne-Legendre indicator value incorporates taxon abundance, it is most valuable when the identified indicator taxon's *presence* is strongly correlated with the environment or community characteristics of interest, as indicated by its  $A_{ind}^g$  and  $B_{pa}$  components as described above; therefore, it is not ideal for identifying taxa that could also be used as an indicator of the impending decline but are relatively ubiquitous and exhibit changes in prevalence, rather than presence in relation to the cycle. To address this limitation, we modelled individual OTU prevalences as a function of phase, sex, season, and location, with the aim of identifying taxa whose prevalences fluctuate with phase. We focused specifically on bacterial taxa whose prevalence are higher in late high populations. Prevalence of each OTU was modelled using a binomial generalized linear model with logit link, weighted by sampling session sample sizes, and fit via maximum likelihood. Models were selected via AIC and further simplified using the *dropterm* function of the *MASS* package; if removal of a term did not increase the AIC value more than 2 greater than the lowest AIC value, then the simplified model was retained. Of those, OTUs in which phase was retained as a term in the most simplified model, and



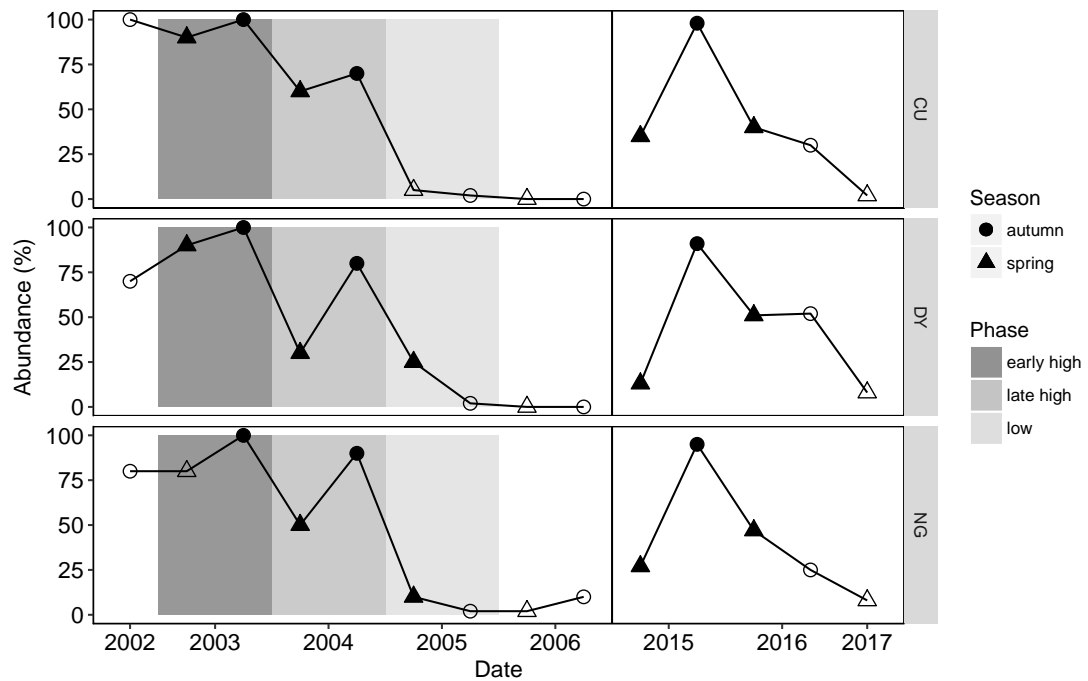
**Fig. 4.2.:** Commune-level abundances of *Arvicola terrestris* from 2002-2016 in Cuvier, Doye, and Onglières in Franche-Comté, France. Abundances range from 0 - no *A. terrestris* observed in the commune to 5 - all parcels colonized by *A. terrestris*. Gaps in the ribbons indicate missing data.

prevalence was higher in late high populations, were selected as potential indicators for comparison with 2015-2016 data.

Prevalences of the OTUs selected via modelling, and those with statistically significant  $IndVal_{ind,max}^g$  with  $A_{ind}^g$  and  $B_{pa}$  over 0.75 (high predictive power and high specificity) were plotted against time and visually inspected for similar patterns of presence/absence in the 2015-2016 samples. Where the selected OTUs were detected in the 2015-2016 data, prevalence of those OTUs in the 2015-2016 data was modelled using a binomial generalized linear model with logit link to determine if prevalence significantly varied with year and season.

### 4.3 Results

Commune-level abundances were not available for all years between 2002 and 2016 in the three locations, but from the data that were available it is clear that after 2004 *A. terrestris* abundances declined in all three locations, peaked again in 2009-2010 and were relatively low between 2011 and 2014 before they increased again (Figure 4.2). Thus, *A. terrestris* populations in these three communes underwent two periods of low population abundance between the two sampling periods.



**Fig. 4.3.:** Transect-based abundance estimates of *Arvicola terrestris* from 2002-2006 and 2015-2016 in Cuvier, Doye, and Onglières in Franche-Comté, France. Seasons are indicated by symbols, periods in which animals were trapped are solid symbols, and periods when transects were conducted but no animals were trapped are shown with open symbols. Phases of the cycle are indicated with shaded rectangles

Local transect abundances were estimated from 2002-2006 and 2015-2017 and ranged from 10 % to 100 % in 2002-2006, and from 13 % to 98 % in 2015-2016 (Figure 4.3.) 2003-2005 sampling session sample sizes ranged from 14 to 31 animals with a sex ratio ranging from 30 % male (Onglières, spring 2005) to 57 % male (Doye, autumn 2004). 2015-2016 sampling session sample sizes ranged from 30 to 37 animals, with a sex ratio ranging from 24 % (Cuvier, autumn 2015) to 56 % male (Onglières, spring 2015). No animals were collected from Onglières in spring 2003 or from Cuvier in spring 2005.

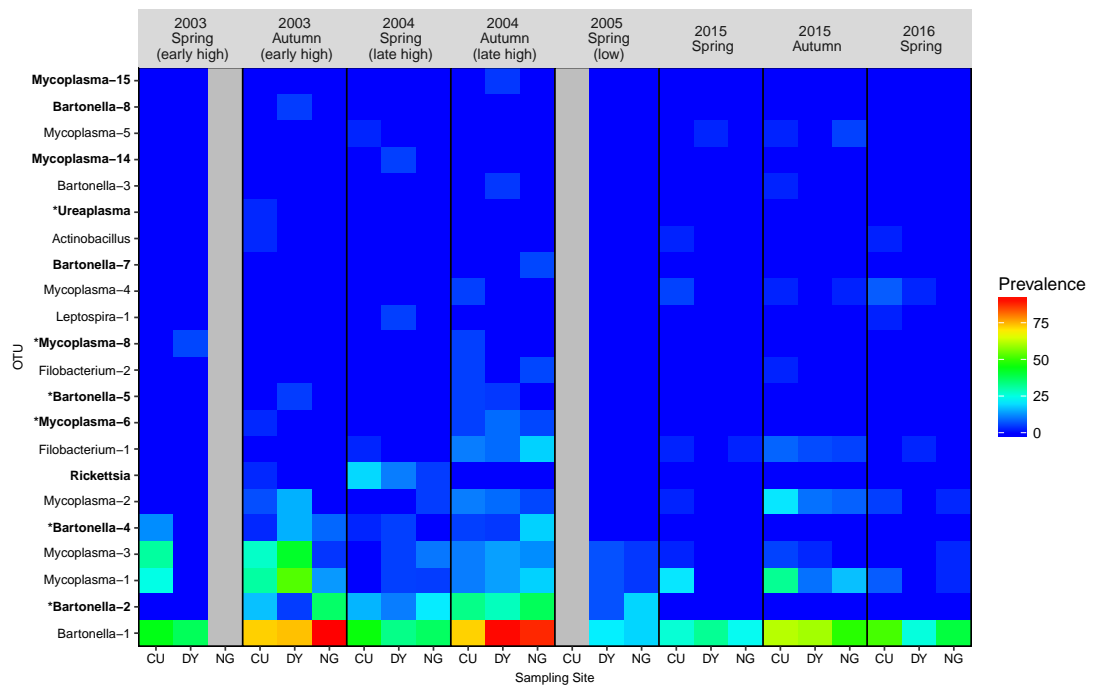
A total of 22 OTUs, assigned to genera whose members include parasites, were identified in 2015-2016 and 2003-2005 spleen assemblages in Cuvier, Doye and Onglières, with prevalences within sampling sessions ranging from 0 to 91 % (Figure 4.4). 10 were identified as *Mycoplasma*, 7 were identified as *Bartonella*, 2 were identified as *Filobacterium* (formerly cilia-associated respiratory (CAR) bacillus), and the remainder were identified as *Ureaplasma*, *Actinobacillus*, *Rickettsia*, and *Leptospira*. 11 of the OTUs detected in the samples from 2003-2005 were not found in 2015-2016 samples (Table 4.2); however, of these 11, 6 were detected at low prevalences in other organs of animals collected in 2014-2016 (Figure 3.2, page 50), thus they haven't completely disappeared from the parasite community. All OTUs found in 2015-2016 samples were detected in 2003-2005 samples.

**Tab. 4.2.:** Mean population prevalence (in %) and standard deviation of OTUs found in spleens collected from *A. terrestris* individuals captured in the communes of Cuvier, Doye, and Onglières in 2003-2005 and 2015-2016. OTUs are ordered by increasing mean prevalence in 2003-2005 sampling sessions (as in Figure 4.4). \* indicates that these OTUs were detected in other organs of animals collected in 2015-2016 and therefore are still present in current *A. terrestris* parasite communities in these sites.

OTU	Mean (2003-2005)	SD (2003-2005)	Mean (2015-2016)	SD (2015-2016)
Mycoplasma-15	0.40	1.30	0	0
Bartonella-8	0.40	1.50	0	0
Mycoplasma-5	0.20	0.90	1.30	2.10
Mycoplasma-14	0.40	1.50	0	0
Bartonella-3	0.40	1.30	0.30	1.00
Ureaplasma	0.30	1.00	0*	0
Actinobacillus	0.30	1.00	0.60	1.20
Bartonella-7	0.50	1.70	0	0
Mycoplasma-4	0.40	1.50	2.60	2.90
Leptospira-1	0.40	1.50	0.30	0.90
Mycoplasma-8	0.90	2.20	0*	0
Filobacterium-2	0.90	2.20	0.30	1.00
Bartonella-5	1.20	2.30	0*	0
Mycoplasma-6	1.90	3.20	0*	0
Filobacterium-1	3.30	6.00	3.40	3.20
Rickettsia	3.00	5.90	0	0
Mycoplasma-2	4.20	5.40	5.60	6.70
Bartonella-4	6.10	6.30	0*	0
Mycoplasma-3	13.20	12.80	1.70	2.20
Mycoplasma-1	14.60	14.70	10.20	11.20
Bartonella-2	17.90	13.10	0*	0
Bartonella-1	55.70	26.30	41.10	14.90

$IndVal_{ind,max}^g$  was sensitive to group resolution for some, but not all, of the OTUs found to have at least one statistically significant  $IndVal_{ind,max}^g$  in the late high-abundance phase, or a smaller group scale within the late high abundance phase (Figure 4.5, Table 4.3); *Bartonella* - 1 and - 2, *Filobacterium* - 1, *Mycoplasma* - 2 and *Rickettsia*  $IndVal_{ind,max}^g$  all increased by at least 49 % when sexes were pooled within season and phase, and this is entirely due to increasing  $A_{ind}^g$ . We also identified some OTUs with significant  $IndVal_{ind,max}^g$  at only one grouping scale; *Bartonella* - 5 and *Filobacterium* - 2 had statistically significant  $IndVals$  at only the phase-season-sex and phase-season scales, respectively. At other scales these OTUs had  $IndVal_{ind,max}^g$  in a late high-abundance group but were not statistically significant.

We selected OTUs with a  $A_{ind}^g$  and  $B_{pa}$  value of at least 0.75 at a resolution of phase-season to compare with samples collected in 2015-2016; this corresponds to a positive predictive power of 0.75 and a sensitivity of 0.75, or more plainly, if we detect the selected OTU in a new site we are at least 75 % confident that the new site is from a late high-abundance phase. This is a high threshold given that we detected statistically significant  $IndVal_{ind,max}^g$  that had  $A_{ind}^g$  and  $B_{pa}$  lower than 0.75; for example, *Bartonella* - 2 had an  $IndVal_{ind,max}^g$  of 0.4 and  $A_{ind}^g$  and  $B_{pa}$  of 0.4 and 1 at the group level of season



**Fig. 4.4.:** Prevalences of parasitic splenic OTUs in 2003-2005 and 2015-2016 populations of *Arvicola terrestris* in Cuvier, Doye, and Onglières in Franche-Comté, France. OTUs are ordered by global occurrence and OTUs that appear only in the 2003-2005 data set are in bold. The asterisk indicates that the OTU was found in samples collected in 2014-2016 in other organs.

and phase. If we used *Bartonella - 2* as an indicator species with the  $IndVal_{ind,max}^g$  method, we would have predicted, with a confidence of 40 %, the decline as happening in 2004 for all three locations instead of in 2005 as it actually did. This highlights the importance of choosing an appropriate threshold in relation to the question being asked; in our scenario, where the phenomenon in question is a distinct ecological event with significant human and economic consequences, a high threshold and the resulting high certainty in prediction is necessary.

*Rickettsia*, *Mycoplasma - 9*, and *Filobacterium - 1* at the phase-season group level were selected as the "best" indicator values because at this scale their  $IndVal_{ind,max}^g$  are above 0.75 and this is the most efficient sampling level - sampling of males and females from a single season can be used with these indicator species.

Only one of the 22 OTUs modelled exhibited prevalences that peaked in the late high phase, *Bartonella - 2*. Table 4.4 shows the model terms, degrees of freedom, deviance and AIC values for the two best models for *Bartonella - 2* prevalence; model (2) was selected as the best and its parameters are shown in Table 4.5. Phase and season were both included in model (2) and therefore phase and season was selected as the "best" resolution with which to compare this OTU's prevalence in 2003-2005 and 2015-2016 data.

**Tab. 4.3.:** Statistically significant  $IndVal_{ind,max}^g$  and their  $A_{ind}^g$  and  $B_{pa}$  components and p-values for bacterial OTUs obtained from the spleens of *Arvicola terrestris* collected from Doye, Onglières and Cuvier, Franche-Comté, France, in spring and autumn 2003-2005, at three different scales of grouping. Sex is indicated by letters (M = male, F = female), and p-values were estimated by permutation (1000 iterations).

Scale	OTU	group	$A_{ind}^g$	$B_{pa}$	$IndVal_{ind,max}^g$	p-value
Phase - Season - Sex	Bartonella-1	late high - autumn - M	0.17	1.00	0.17	< 0.01
	Bartonella-2	late high - autumn - M	0.27	1.00	0.27	< 0.01
	Bartonella-5	late high - autumn - F	0.69	0.67	0.46	< 0.01
	Filobacterium-1	late high - autumn - M	0.63	1.00	0.63	< 0.01
	Mycoplasma-2	late high - autumn - M	0.27	1.00	0.27	0.02
	Mycoplasma-6	late high - autumn - M	0.84	1.00	0.84	< 0.01
Phase - Season	Rickettsia	late high - spring - F	0.59	1.00	0.59	< 0.01
	Bartonella-1	late high - autumn	0.32	1.00	0.32	< 0.01
	Bartonella-2	late high - autumn	0.40	1.00	0.40	< 0.01
	Filobacterium-1	late high - autumn	0.92	1.00	0.92	< 0.01
	Filobacterium-2	late high - autumn	1.00	0.67	0.67	0.03
	Mycoplasma-2	late high - autumn	0.49	1.00	0.49	0.03
Phase	Mycoplasma-6	late high - autumn	0.86	1.00	0.86	< 0.01
	Rickettsia	late high - spring	0.91	1.00	0.91	< 0.01
	Filobacterium-1	late high	1.00	1.00	1.00	0.02
	Mycoplasma-6	late high	0.82	1.00	0.82	0.02
	Rickettsia	late high	0.90	1.00	0.90	0.01

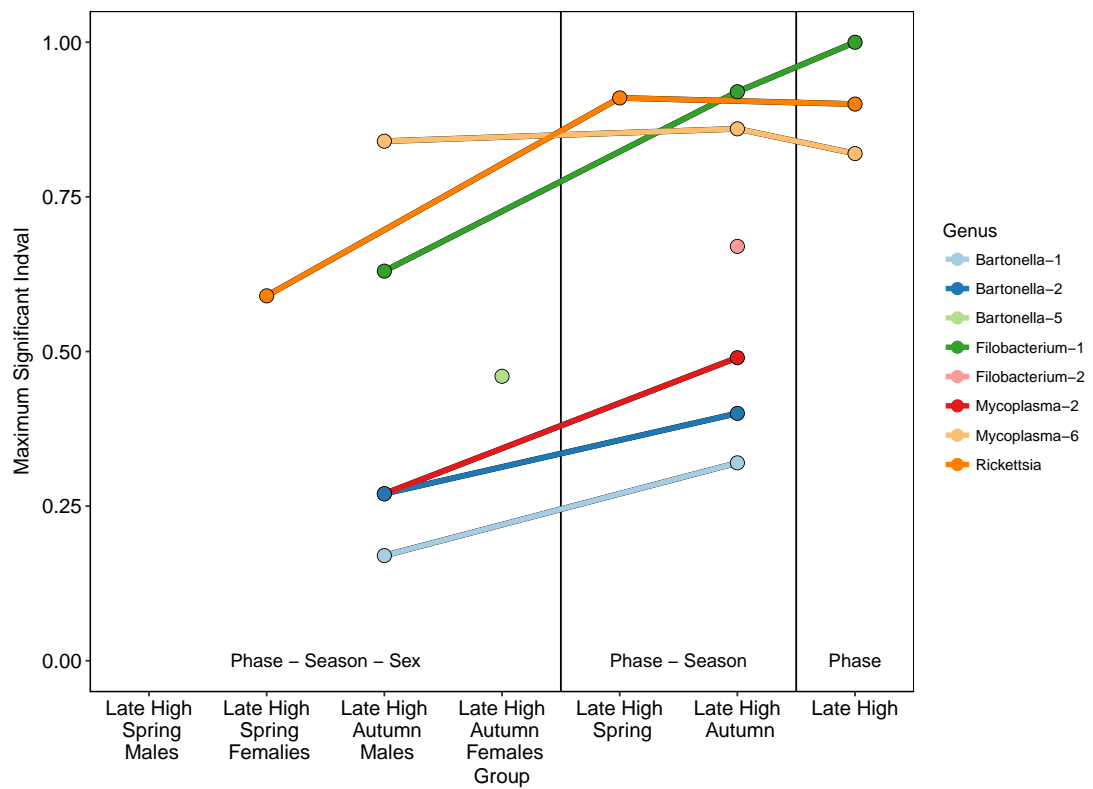
**Tab. 4.4.:** Model terms, degrees of freedom (df), Deviance, AIC values, and  $\Delta$ AIC values for the 2 models with the lowest AIC scores for *Bartonella* - 2 prevalence in spleens collected from *Arvicola terrestris* in springs and autumns of 2003-2005 in Doye, Onglières, and Cuvier, Franche-Comté, France. Models were fit using a binomial generalized linear model with a logit link, with maximum likelihood.

	Model Terms	df	Deviance	AIC	$\Delta$ AIC
(1)	sex + phase + season + location	6	15.67	76.95	0.44
(2)	phase + season + location	5	17.24	76.51	0

Figure 4.6 shows the prevalences of the 4 selected OTUs in the 2003-2005 and 2015-2016 data, at the resolution of phase and season. Only 1 appears in the 2015-2016 spleen samples: *Filobacterium* - 1; the remaining three, *Bartonella* - 2, *Rickettsia*, and *Mycoplasma* - 6, were not detected in the 2015-2016 spleens. Prevalences for *Filobacterium* - 1 in 2015-2016 samples ranges from 0 to 8.8 % (0-17.8 % 95 % confidence interval) with higher prevalences (though not significantly so) found in all three locations in autumn 2015 (Table 4.6).

**Tab. 4.5.:** Parameters and estimates (untransformed) for model (2) from Table 4.4.

Parameter	Estimate	Standard Error	P-value
Intercept	-1.62	0.37	< 0.0001
Late High	1.00	0.39	0.011
Low	0.84	0.71	0.23
Spring	-1.28	0.41	0.001
Doye	-0.53	0.45	0.24
Onglieres	0.61	0.40	0.24

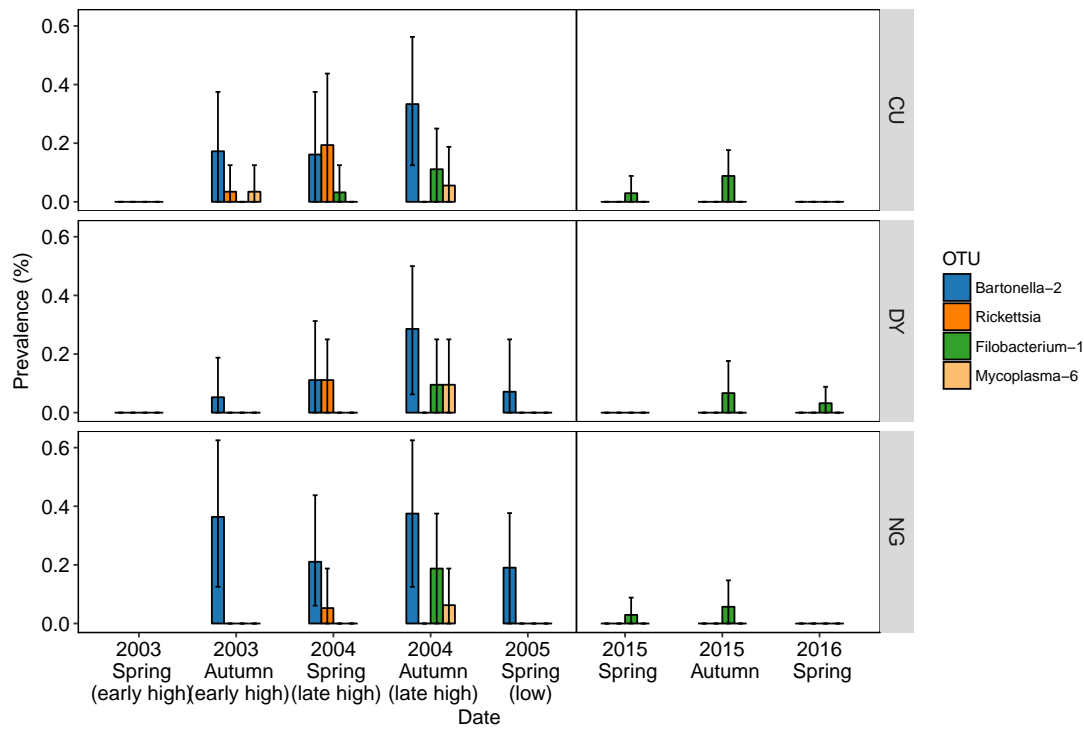


**Fig. 4.5.:** Maximum statistically significant IndVals at different scales of grouping, from broad (phase) to specific (phase, season, sex).

**Tab. 4.6.:** Parameters and estimates (untransformed) for *Filobacterium* - 1 prevalence in current sampling sessions. Model was fit using a binomial generalized linear model with a logit link, with maximum likelihood.

Parameter	Estimate	Standard Error	P-value
Intercept	-2.58	0.39	< 0.0001
Spring 2015	-1.31	0.81	0.11
Spring 2016	-1.99	1.08	0.06





**Fig. 4.6.:** Prevalences of the 3 OTUs with statistically significant high ( $>0.75$ ) maximum IndVals for late high-abundance autumn sampling sessions in Cuvier (CU), Doye (DY), and Onglières (NG). 95 % confidence intervals were estimated via bootstrapping.

## 4.4 Discussion

We present here the first investigation into parasitic bacteria communities from different *A. terrestris* outbreaks in the Franche-Comté region of eastern France. We have found that splenic bacterial parasite communities collected from the same populations 10 years apart have moderately similar composition; half of the OTUs detected in 2003-2005 were present in samples from 2015-2016, and of the OTUs not detected in 2015-2016 spleens, 6 were still present in these populations, just detected in other organs. We did not observe any new OTUs in 2015-2016 that were not present in spleens from 2003-2005.

The loss of rare OTUs (e.g. *Mycoplasma*-15, *Bartonella*-8) is not surprising; rare OTUs are more vulnerable to stochastic extinction events because the absolute number of hosts supporting them is smaller (Poulin and Morand, 2004; MacArthur and Wilson, 1967). Relative isolation between host populations during the low-abundance phase (as measured by gene-flow, Berthier et al., 2006) may also contribute to OTUs dropping out of the host populations, because rescue-like effects are less likely to occur. Together, these processes might have contributed to the loss of some of the rare OTUs detected in 2003-2005. Genetic isolation observed in *A. terrestris* populations at low density is not always representative of isolation between parasite populations though (Deter et al.,

2007), and further investigations into individual OTU prevalences and host dispersal should be investigated.

Why new bacterial OTUs weren't detected in 2015-2016 is not clear, but may be related to host abundance; the outbreak in 2003-2005 was characterized by persistent high abundance at the landscape level from 2002 to 2004, while the outbreaks in 2009-2010 and 2015-2016 show more variation (Figure 4.2). Invasibility of a host population is expected to increase with increasing host abundance, and longer periods of high abundance may act analogously to island age *sensu* island biogeography theory, accumulating more species as time passes (Poulin and Morand, 2004; Kuris et al., 1980). If some of the parasites detected in 2003-2005 are "spillover" infections from other host species, their disappearance may be due to reduced invasibility of *A. terrestris* populations at low abundance or short periods of high abundance. Further investigations into the role other host species play in the parasite community dynamics of *A. terrestris* is necessary to better assess this possibility.

Vector transmission may also play a role in the results we have obtained; for example, *Rickettsia* is found in ticks *Ixodes ricinus* and *Dermacenter* sp. (Špitalská et al., 2008; Mediannikov et al., 2008) in Europe, and while its transmission between rodent hosts and tick vectors is not clear (Pluta et al., 2010; Burri et al., 2014), tick distribution and behaviour can vary with season as both population structure and local climate conditions change (Daniel et al., 2015; Randolph et al., 2002). This may play a role in *Rickettsia* distribution in host populations, and may have contributed to the apparent disappearance of *Rickettsia* from 2015-2016 spleens.

The decline of *Bartonella*-2 is particularly interesting given that it was the second-most prevalent OTU in 2003-2005 and had disappeared from spleens in 2015-2016. Bacterial parasites in other systems exhibit complex dynamics including competitive and facilitative interactions; Telfer et al. (2008) reported that in *Microtus agrestis* populations, *Bartonella taylorii* infection duration was reduced when animals were co-infected with *Babesia microti*, illustrating one way in which parasites may influence each-other's persistence in the host population. *Bartonella* species can also vary dramatically in terms of their seasonal and host dynamics; Telfer et al. (2007) reported significant differences in seasonal dynamics, density dependence, and host prevalence between four species of *Bartonella* coexisting in bank voles and wood mice. Thus differences in how OTU prevalences have changed may also be related to parasite species characteristics - further investigations into the strains of bacteria we have detected may prove informative in this matter.

Given that we have only data from 2015-2016 and not the complete cycle, it would be premature to make in-depth comparisons between the 2003-2005 communities and 2015-2016 communities, but in general, the broad pattern of persistence of a portion

of the splenic bacterial parasite community within the host population over a period of over a decade is consistent with the few other long-term studies on bacterial parasites in rodents. Alsarraf et al. (2016) found that while prevalences of various haemoparasites (*Babesia*, *Bartonella*, *Mycoplasma*, *Hepatozoon*, *Trypanosoma*) in spiny mice (*Acomys dimidiatus*) in Egyptian wadis changed, sometimes dramatically, over a period of 12 years, composition of the community was relatively stable with only one of the five genera dropping out by the end of the study period. The same haemoparasite communities in bank voles in Poland showed similar compositional stability over a period of 11 years (Bajer et al., 2014). Both of these studies limited their analyses to the genus level and thus it is unclear if there was any turnover or significant changes within genera, but they suggest that even in the relatively isolated rodent populations they studied, parasitic bacteria genera can persist within rodent populations for over a decade.

We have identified several potential bacterial parasite indicators of the late high-abundance phase in *A. terrestris* spleens collected from Doye, Onglières and Cuvier in 2003-2005 using the group-corrected *IndVal* metric applied to several different scales, with *Indvals* calculated at moderate group resolution being selected as "best" due to high predictive power and sensitivity of the OTUs selected coupled with the most efficient sampling scheme. We have also identified one potential indicator by modelling its prevalence as a function of cycle phase. Only one of these indicators (*Filobacterium*-1) was detected in spleens collected from the same locations in 2015-2016.

*Filobacterium* sp., formerly cilia-associated respiratory bacillus, has only ever been isolated from lung tissue (Nietfeld et al., 1999; Nietfeld et al., 1995; Bergottini et al., 2005). Our results may therefore be due to infections in which the bacteria have penetrated the lung epithelium and entered the bloodstream, or the accumulation of inert *Filobacterium* DNA in the spleen. In wild rats, *Filobacterium* can be isolated from diseased and clinically normal animals (Brogden et al., 1993), which may be evidence that it's a normally benign component of some respiratory microfloras. Why this particular OTU, of the 4 detected in 2003-2005 samples, persisted in 2015-2016 is unclear, but it may be related to it's supposed niche within the host. *Rickettsia*, as discussed above, is vector-borne and it's absence in 2015-2016 samples may be related to tick distribution in the landscape. *Bartonella* and some *Mycoplasma* are parasites of erythrocytes (Browning et al., 2010; Schülein et al., 2001) which may result in more direct competitive interactions between these two genera, increasing their chances of dropping out of the host.

The application of the *IndVal* metric to identify indicator species for ecological and environmental phenomena is well established (Siddig et al., 2016), but its application to parasites specifically is less common. Antonelli et al. (2015) used the *IndVal* metric to identify a parasitic flatworm, two copepod species, an isopod and a myxosporidian which acted as indicators of the season or location from which their farmed European

sea bass *Dicentrarchus labrax* hosts were collected. Kaouachi et al. (2014) identified 6 monogenean species that were significant indicator species for the location from which their teleost fish hosts were collected, and 8 monogenean species that were significant indicator species for the host species from which they were collected. Quilchini et al. (2010) identified several nematodes and trematodes that were significant indicators of altitude, watershed and the season during which their brown trout hosts were collected. In all three cases, parasite burden on individual hosts could be established and used as abundance during the calculation of the *IndVal*; individual hosts were treated as sites, unlike our approach in which populations were treated as sites and we used prevalence within the population instead of parasite burden. Some studies using high-throughput sequencing have used read numbers (the number of amplicons of an OTU detected) as a proxy for taxon abundance (e.g. Gomez et al., 2016) but this practise is controversial as read numbers do not always strongly correlate with independent estimates of taxon abundance (Amend et al., 2010).

Investigations into grouping sites or species when calculating *IndVal* metrics has been conducted in the past (De Cáceres et al., 2010; De Cáceres et al., 2012), but to our knowledge this is the first investigation into forming groups based on sex or time periods to calculate *IndVal*. That some OTU *IndVal* (e.g. *Filobacterium*-1 or *Mycoplasma*-2) were sensitive to changes in scale is due to how they were distributed in the population: at smaller group sizes, these target OTUs are found in more than one group (i.e. they are found in both sexes within a location, season, and phase); pooling the sexes together increases the "concentration" of these OTUs in the target group by making the target group more inclusive and reducing the proportion of non-zero prevalences outside of the target group. For *Mycoplasma* - 6, that was not as sensitive to changes in group scale, pooling sexes or seasons together to make larger groups had little effect on  $IndVal_{ind,max}^g$  or in fact decreased it slightly because the aggregation of season or sex did not increase the concentration of this OTU in the target group. Numerically, pooling sites together to create larger groups and then calculating the prevalence of the target OTU is equivalent to calculating a weighted mean of the prevalences of the target OTU in the sites before they are pooled, with weights corresponding to the number of animals in each site; this can lead to slight decreases in the pooled  $A_{ind}^g$  if sites with more animals have lower prevalences, as it drags the new pooled prevalence down.

We've presented here a cursory look at how parasitic bacterial communities vary across iterations of the *A. terrestris* cycle, and a preliminary investigation into identifying one or more bacterial taxa that could be used to indicate the late high-abundance phase of *A. terrestris* population outbreaks. That we identified several taxa with statistically significant *IndVal* for the late high-abundance phase is exciting, but as the value of an indicator lies in the strength of its correlation with the phenomenon of interest (National Research Council, 2000; McGeoch and Chown, 1998), our current results are not particularly encouraging. Analyzing the spleens of animals collected in 2016-2018 is

imperative for fully exploring how splenic bacterial parasite communities have changed since 2003-2005, and assessing whether any of the indicator taxa identified for the 2003-2005 cycle are useful for future outbreaks.

### Summary

- Splens from 2003-2005 harboured 22 bacterial parasite OTUs
- Splens from 2015-2016 harboured only 11 of those 22 OTUs found in splens from 2003-2005
- 6 of the 11 "missing" OTUs from 2015-2016 were detected in other organs collected from that period, thus they were not lost entirely from the regional pool
- Four OTUs were identified as indicators of the late high-abundance phase of the 2003-2005 outbreak
- Only one of these four OTUs was detected in splens from 2015-2016
- long term heterogeneity in splenic bacterial parasite community composition in *A. terrestris* was characterized by the loss of bacterial OTUs
- bacterial indicators of the late high-abundance phase could be identified in the 2003-2005 outbreak, but their application to the 2015-2016 outbreak is currently unclear



## Conclusions and Future Directions

### 5.1 Thesis Objectives Revisited

We present here the first detailed study of bacterial parasite communities in fossorial water vole *Arvicola terrestris* populations in the Franche-Comté region of eastern France. Bacterial parasites represent one component of the parasite community that has received relatively little attention in *A. terrestris* in this region, but bacteria are likely to be an important component of this system for a number of reasons, including their interactions with other parasites like viruses (Telfer et al., 2010) and helminths (Jolles et al., 2008), and their potential risk to humans. In addition, bacterial parasite communities, and parasite communities in general, have not been examined in the context of rodent population cycles, a phenomenon that has proven resistant to elucidation for almost a century (Krebs, 2013). Given the paucity of information on bacterial parasites in this region, the development of high-throughput DNA sequencing and metabarcoding approaches that allow for rapid identification of bacterial species in a large sample set, and interest from the local agricultural community in developing management tools, we have attempted to characterize the spatial and temporal variation in bacterial parasite communities in fluctuating *A. terrestris* populations, in order to produce a sound foundation upon which further investigations into the bacterial parasite community can be based.

Our first question was a technical one regarding the use of 16s rRNA bacterial gene sequencing to detect parasites in rodent hosts: which rodent organ should be sampled? Spleens have been used in other studies (Razzauti et al., 2014) due to their role in the immune system and clearing pathogenic bacteria from the body (reviewed by Altamura et al., 2001), but some bacteria are known to target other organs and may be missed if only the spleen is used (e.g. *Leptospira* targets the kidney, Adler and de la Peña Moctezuma, 2010). The results of the pilot study we conducted to answer this question, in which we compared the bacteria detected in the heart, liver, lungs, spleen and kidneys from 13 male *A. terrestris*, suggests that including additional organs increases the detection power; more potentially pathogenic bacteria OTUs were detected within an animal when more organs were considered, with maximum coverage of the bacterial community only obtained when all five organs were used. It is unclear though if this result is actually driven by using difference organs, or simply due to resampling an animal five times.



Our remaining questions related to spatial and temporal heterogeneity in parasite communities in *A. terrestris* at the individual host (infracommunity) scale and the host population (component community) scale. Infracommunity richness is thought to be the result of the colonization rate of new parasites and the duration of subsequent infections (Poulin, 1995), thus intrinsic factors like immunocompetence and age, and extrinsic factors such as population density (as a correlate of contact rate) may all be expected to play a role in infracommunity richness. We addressed this question in Chapter 3, and found that infracommunity richness was best predicted by location and date, with animals collected in autumn having richer infracommunities than spring animals. PERMANOVA analysis indicates that infracommunity composition is highly heterogeneous, even within populations, and other factors like host sex explain little of the variation observed. Infracommunity similarity was weakly but significantly correlated with geographic proximity for each of the four sampling periods considered.

We asked similar questions regarding component community richness and similarity; work in parasite ecology suggests that population density may drive patterns of component community richness (Poulin, 2007), and increases in effective *A. terrestris* dispersal between populations, as measured by gene flow, over the peak of an outbreak (Berthier et al., 2005; Berthier et al., 2006) may be expected to homogenize parasite communities during the high abundance phase. We found that component community richness is best predicted by current local abundance, with higher richness observed in populations at higher abundance, and PERMANOVA analysis detected significant temporal effects in component community similarity (component communities from the same sampling period were more similar to each other than to component communities from other sampling periods), suggesting that local *A. terrestris* component communities are not strongly isolated. Geographic distances between sites and component community similarity were not correlated, which further supports the conclusion that at moderate scales (20km) *A. terrestris* parasite component communities are not independent.

We were also interested in longer-term temporal variability; other studies on *A. terrestris* parasites in this region have been restricted to a single outbreak, but these periodic and dramatic fluctuations in abundance might have significant effects on the bacterial parasite community composition as local *A. terrestris* populations experience fluctuations in connectivity (Berthier et al., 2005; Berthier et al., 2006). We addressed this question in Chapter 4, and determined that there is moderate turnover in bacterial OTUs over a period of 13 years; 11 OTUs of the 22 detected in *A. terrestris* spleens in Cuvier, Doye and Onglières in 2003-2005 were detected in the spleens of animals collected from those same locations in 2015-2016, and of the 11 that were not detected in the most recent samples, 6 were detected in other organs of the animals tested. Interestingly, the 2<sup>nd</sup> most prevalent OTU in 2003-2005 is one of the OTUs that disappeared from spleens. Thus, changes in bacterial regional community composition over the long term

are not restricted to turnover in rare OTUs but also include the significant decline of one bacterial OTU that was a dominant member of the community in 2003-2005.

We identified several significant indicators of the late high-abundance phase of 2003-2005 using the *IndVal* approach and generalized linear modelling; however, only one of the four indicator OTUs for 2003-2005 were present in 2015-2016. We refrain from rejecting this approach for identifying a predictor of the decline phase outright due to an incomplete data set, but we recognize that a failure to detect three of the four 2003-2005 indicators in 2015-2016 populations is reminiscent of some of the earliest work on disease in cycling populations, in which single parasites were rejected as causes of the decline (Chitty, 1954), and we emphasize that other aspect of the parasite community need to be assessed.

## 5.2 Caveats and Limitations

### 5.2.1 High-Throughput Sequencing

High-throughput sequencing using metabarcoding and multiplex approaches are becoming more and more common in microbial ecology research, but they have limitations. As discussed in Chapter 2 of this thesis and the accompanying published article, metabarcoding multiplex approaches for identifying bacterial parasites in rodents have sometimes relied on only one or a few host organs (e.g. Razzauti et al., 2014; Galan et al., 2016; Cosson et al., 2015; Koskela et al., 2017; Rossow et al., 2014), and we have demonstrated that including additional organs in the analysis increases coverage of the bacterial parasite community (Villette et al., 2017). We have not, however, eliminated the possibility that our results are due to resampling and not to actual tissue identity; i.e., if we had sampled the same spleen five times, instead of the spleen, heart, liver, kidney and lungs, would we have seen a similar increase in community coverage? Given that the spleen is a critical organ for clearing bacterial infections, perhaps; Razzauti et al. (2015) isolated *Leptospira* DNA from *Myodes glareolus* spleens despite pathogenic leptospires typically inhabiting kidney tissue (Adler and de la Peña Moctezuma, 2010). In addition, for the analyses presented in Chapters 3 and 4 we pooled four of the five organs together during the DNA extraction process; this may have resulted in a dilution effect, in which pooled-organ bacterial OTU richness is in fact lower than if we had analyzed each organ separately, and this also warrants further study.

Potential bias in metabarcoding approaches may also influence coverage of the bacteria parasite community; one of the clearest examples demonstrating such bias is presented by Razzauti et al. (2015), in which they compared rRNA transcriptome sequencing with high-throughput sequencing of the 16s rRNA gene using the 454 pyrosequencing

platform and the MiSeq Illumina platform. In general, they found broad overlap in the bacterial species detected using the rRNA transcriptome sequencing approach and 16s rRNA sequencing with the MiSeq Illumina platform; however, there were several taxa that were entirely missed by 16s rRNA sequencing - notably *Francisella* sp. and *Coxiella* sp., both of which we might expect to see in *A. terrestris* in Franche-Comté. (see Box 5.1 for details)

**Box 5.1: *Francisella* sp. and *Coxiella* sp.**

*Francisella tularemia* is a vector-transmitted species (Mörner and Addison, 2001), and has been detected in other *A. terrestris* populations including in Germany (Kaysser et al., 2008), Ukraine (Hightower et al., 2014) and other former Soviet Union countries (Pollitzer, 1967). Antibodies to *F. tularemia* were found in 40 % of forestry workers surveyed in Franche-Comté (Rigaud et al., 2016), indicating that this parasite is present in the region. That *F. tularemia* can be found in other small mammals in Europe including *Microtus* sp., *Apodemus*, and *Lepus* sp. (World Health Organization, 2007) may account for the apparent discrepancy between its presence in humans and its apparent absence in *A. terrestris* in Franche-Comté, but further screening for this parasite in the samples we have collected is necessary to confirm this.

*Coxiella* has been isolated from *A. terrestris*, *Microtus* sp. and *Apodemus* sp. in the former Czechoslovakia (Meerburg and Reusken, 2011) but there are no reports on its prevalence in ticks or other animals in Franche-Comté, and no evidence of infections in humans in this region either (Frankel et al., 2011; Serre et al., 2017); therefore its absence in our data set could be due to its absence in *A. terrestris* or the region rather than bias in the methods used here, but further analysis to confirm this result would be prudent.

Sequencing the V4 region of the 16S rRNA gene, while not unusual for studying bacterial communities (e.g. Caporaso et al., 2012; Shepherd et al., 2011; Razzauti et al., 2014), also presents some limitations. Copy numbers of the 16s gene within bacterial taxa vary from as low as 1 copy per genome to 15, which is likely to bias abundance estimates based on sequence abundance (Větrovský and Baldrian, 2013). We don't attempt to quantify parasite load in individuals using sequence abundances, but we still face potential bias because the copies in taxa with more than one copy are not always identical, which may result in the sequences of one species being sorted into multiple OTUs (Větrovský and Baldrian, 2013). In addition, the V4 region is suitable for distinguishing between bacterial genera but can be somewhat conserved between species within the same genus, limiting its utility for precise characterization of bacterial communities (Chakravorty et al., 2007). Given that some bacterial genera can be very diverse in terms of their propensity for causing disease in hosts (see Appendix regarding *Mycoplasma*, for example), further analysis to identify species is necessary.

## 5.2.2 Bacterial Parasites

As described in Chapter 2 we selected only those bacteria that have limited survival and reproduction outside of the host (obligate parasites), and we also eliminated bacteria typically found in the gut. Koskela et al. (2017), also using a DNA sequencing approach, reported a number of bacterial species typically localized to the gut in the livers of *Microtus agrestis*, *Microtus oeconomus*, and *Myodes glareolus*, including members of *Clostridiales*, *Lachnospiraceae* and *Ruminococcaceae*. Razzauti et al. (2014) reported a similar trend with *Helicobacter* sp, typically thought to be restricted to the gut, appearing in rodent spleen samples. Koskela et al. (2017) speculated that the presence of nominally gut bacterial DNA in the liver could be due either to a weakening of the intestinal wall during the early stages of decomposition (the voles they sampled were snap-trapped overnight), or non-viable remnants of bacteria accumulating in the liver. However, gut bacteria can penetrate the intestinal epithelium and enter the blood stream, typically in response to disruptions in the normal gut flora following shock or injury (reviewed by Balzan et al., 2007). Distinguishing between translocated bacteria, non-viable remnants of bacterial DNA, and contaminants of the dissection process is one area that requires more work - DNA sequencing of the murine gut flora in conjunction with antibody screening may be one way to identify translocated gut bacteria (e.g. Maes et al., 2012).

Gut bacteria are not the only bacteria that may be opportunistic pathogens; environmental bacteria (i.e. those found as normal components of the water or soil microbial community) can also infect immunocompromised individuals. For example, *Acinetobacter baumannii* is a common soil bacteria that is also a major cause of nosocomial infections in human and veterinary hospitals (Bergogne-Berezin and Towner, 1996; Francey et al., 2000). Information regarding opportunistic infections in wildlife populations is sparse, but they may contribute to infracommunity and component community structure if they increase host susceptibility to infection by other parasites. Such infections may be particularly important in the context of declining body condition and immunocompetence during the decline phase (Charbonnel et al., 2008b).

In addition, the methods we have used here to quantify richness and community similarity make no distinction between closely and distantly related taxa, but interactions between closely- and distantly-related taxa may differ and contribute to the patterns that we've seen. Diversity measures that incorporate taxonomic information, like Unifrac (Lozupone and Knight, 2005) may provide a more nuanced picture of how bacterial parasite communities change over time.

## 5.3 The Parasite Community

### 5.3.1 Bacterial Parasites as a Guild

We have focused on the bacterial parasites of *A. terrestris* because relatively little is known about them and high-throughput sequencing offers the opportunity to assess the community as a whole without the *a priori* assumptions regarding membership that are required for other methods (culture or targeted sequencing methods). But *A. terrestris* host a wide variety of other parasites (see Table 5.1 for a list of documented parasites in Franc-Comtoise and other *Arvicola* sp. populations), and interactions between parasites of different guilds in other parasite-host systems are known, although not always well understood (see Cox (2001) for a review). In field voles (*Microtus agrestis*), infection with the bacterium *Anaplasma phagocytophilum* can significantly increase the risk of infection by cowpox virus and the protozoan *Babesia microti*, and infection with cowpox virus can increase risk of infection by *A. phagocytophilum*, *B. microti*, and *Bartonella* sp. (Telfer et al., 2010). Such interactions are not limited to microparasites; macroparasites like helminths appear to play a role in microparasite infection dynamics and community structure in some systems. Jolles et al. (2008) reported a strong negative correlation between infection with bovine tuberculosis (*Mycobacterium bovis*) and gastrointestinal nematodes in African buffalo, and Nunn et al. (2014) reported a strong correlation between helminth prevalence and intracellular parasite richness in primates. Thus, we can expect that other macro- and microparasites in Franc-Comtois *A. terrestris* have contributed to the patterns of infra- and component community structure that we have observed here, and further investigations into the other parasites that our sampled animals host is warranted.

### 5.3.2 Spatial Patterns

We failed to detect a strong effect of distance on infracommunity or compound community similarities, which we have interpreted as evidence that geographic distance is not strongly correlated with actual isolation. Geographically proximate populations are not more similar to each other than geographically distant populations, but also, geographically distant populations still have a moderate degree of overlap in component community composition. Despite being separated by over 10km of forest habitat, Jaccard dissimilarity indices between Arc-sous-Montenot and the other sites ranges from 0.21 to 0.52 (where 0 indicates the communities are identically composed, and 1 means they have no species in common), and Jaccard dissimilarity indices between closer sites ranged from 0.28 to 0.65.

**Tab. 5.1.:** Parasites identified in *Arvicola* sp. in Franche-Comté and elsewhere in Europe.

		Species	Location
Bacteria	Gut parasites	<i>Campylobacter</i> spp.	Scotland †
		<i>Salmonella enterica</i>	Scotland †
		<i>Escherichia coli</i>	Scotland †
	Other	<i>Bartonella</i> spp.	Scotland †
		<i>Leptospira</i> spp.	Scotland †
Viruses	Hantavirus	<b>Puumala virus</b>	<b>Franche-Comté</b> <sup>g</sup>
		Tula virus	Germany, Switzerland <sup>h</sup>
	Orthopox virus	<b>Cowpox virus</b>	<b>Franche-Comté</b> <sup>g</sup>
	Arenavirus	<b>Lymphocytic choriomeningitis virus</b>	<b>Franche-Comté</b> <sup>g</sup>
Helminths	Nematoda	<i>Syphacia nigeriana</i>	Franche-Comté ‡
		<i>Trichuris arvicolae</i>	Franche-Comté ‡
			Scotland †
		<i>Trichuris muris</i>	Franche-Comté <sup>f</sup>
		<i>Capillaria murissilvatici</i>	Franche-Comté <sup>f</sup>
		<i>Capillaria</i> sp.	Franche-Comté <sup>f</sup>
		<i>Heligmosomoides laevis</i>	Franche-Comté <sup>f</sup>
		<i>Aonchotheca</i> sp.	Franche-Comté ‡
		<i>Eucoleus bacillus</i>	Franche-Comté ‡
		Strongyles (unspecified)	Scotland †
	Cestoda larvae	<i>Ecchinococcus multilocularis</i>	Franche-Comté ‡
		<i>Taenia taeniaeformis</i>	Franche-Comté ‡
		<i>Taenia crassiceps</i>	Franche-Comté ‡
		<i>Taenia tenuicollis</i>	Franche-Comté <sup>f</sup>
		<i>Calodium hepaticum</i>	Austria <sup>i</sup>
	Cestoda adults	<i>Anoplocephaloides dentata</i>	Franche-Comté ‡
		<i>Paranoplocephala gracilis</i>	Franche-Comté ‡
		<i>Paranoplocephala omphalodes</i>	Franche-Comté ‡
			Novosibirsk <sup>a</sup>
		<i>Arostrilepis cf horrida</i>	Franche-Comté ‡
		<i>Hymenolepsis</i> spp.	Scotland †
		<i>Aprostataandrya</i> sp.	Franche-Comté <sup>f</sup>
		<i>Aprostataandrya dentate</i>	Novosibirsk <sup>a</sup>
<i>Arostrilepis microtis</i>		Novosibirsk <sup>a</sup>	
<i>Arvicollepis transfuga</i>		Novosibirsk <sup>a</sup>	
<i>Paranoplocephala blanchardi</i>		Novosibirsk <sup>a</sup>	
<i>Parandria feodorovi</i>		Novosibirsk <sup>a</sup>	
Coccidia		Sarcocystidae	<i>Frenkelia glareoli</i>
	<i>Frenkelia microti</i>		Franche-Comté ‡
	<i>Toxoplasma gondii</i>		Franche-Comté <sup>e</sup>
			Scotland †
			Austria <sup>b</sup>
		Austria <sup>b</sup>	
Other Eukaryotes	Cryptosporidiidae	<i>Cryptosporidium</i> spp.	Scotland †
	Metamonada	<i>Giardia</i> spp.	Scotland †
	Apicomplexa	<i>Babesia microti</i>	Scotland †
	Amoebozoa	<i>Entamoeba</i>	Scotland †
Fungi		<i>Emmonsia crescens</i>	England <sup>d</sup>
		<i>Pneumocystis carinii</i>	Auvergne <sup>c</sup>
		<i>Encephalitozoon cuniculi</i>	Austria <sup>b</sup>

†Gelling et al. (2012); ‡Tollenaere et al. (2008); \*Gerlinskaya et al. (2013); <sup>b</sup> Fuehrer et al. (2010); <sup>c</sup>Goyot et al. (1986); <sup>d</sup>Chantrey et al. (2006); <sup>e</sup>Kia et al. (2004); <sup>f</sup>Cerqueira et al. (2007) <sup>g</sup>Charbonnel et al. (2008a); <sup>h</sup>Schlegel et al. (2012); <sup>i</sup>Führer et al. (2010)

What's driving the spatial turnover in component community composition at short distances? Poulin and Morand (1999) suggested that large geographic distances between host populations may contribute to differences in their parasite communities, but local processes may act to generate differences at smaller scales. Local adaptation is one factor that may be important in shaping differences in component communities between adjacent populations. Local adaptation by a parasite species to their host is not uncommon (Greischar and Koskella, 2007), and is expected to occur when parasite gene flow is higher than host gene flow. This may be particularly important during the low phases, when gene flow between neighbouring *A. terrestris* populations is depressed compared to the high abundance phase (Berthier et al., 2006). This was one potential explanation cited by Deter et al. (2007) for explaining why neighbouring populations of *A. terrestris* didn't have similar prevalences of *Trichuris arvicolae*.

Conversely, what's driving the similarities between distant component communities? *A. terrestris* dispersal may be an important driver of regional similarity; though Saucy (1994a) reported that dispersal was rarely over 100 m in Franc-Comtois *A. terrestris*, genetically homogeneous populations in Franche-Comté can be found at scales up to 20 km (Berthier et al., 2005), and Scottish aquatic *Arvicola* may disperse as far as 5 km (Telfer et al., 2003a), thus we cannot eliminate host dispersal as a contributor to regional similarities in component community composition.

For vector-transmitted parasites, direct contact between host individuals is not necessary; vectors may themselves move long distances (e.g. some mosquitos can travel tens of km, Verdonshot and Besse-Lototskaya, 2014), or be transported long distances by other hosts (reviewed by Hubálek, 2004, for birds), thus vectors may serve to drive some of the large-scale patterns in parasite community similarity. In addition, other host species in the system may also contribute to parasite community similarity across larger scales; parasite dispersal patterns are predicted to be driven by the most vagile host (Jarne and Théron, 2001), thus if some of the parasites we have observed in *A. terrestris* here are also found in more vagile species in the region, this may contribute to long-distance similarities in component communities.

### 5.3.3 The Parasite Compound Community

As we have alluded to above, we don't expect *A. terrestris* to be an isolated host species; many of the parasites we have detected are likely capable of parasitizing other hosts, including the mycoplasmas (Pitcher and Nicholas, 2005) and *Bartonella* sp. (Oliver et al., 2009; Birtles et al., 2001); indeed, some of the parasites we have detected here may not even use *A. terrestris* as their reservoir or primary host. In this context in which *A. terrestris* is one of many potential hosts in the ecosystem, then the impact of *A. terrestris* multiannual population fluctuations is not limited to just changes in grassland

community composition (Delattre and Giraudoux, 2009), or population density of predators (Weber et al., 2002), but likely also on the parasite compound community, or all parasites found in the ecosystem.

*A. terrestris* population fluctuations may effect the compound community composition by acting as a source of infections in other host species. For example, *Echinococcus multilocularis* infection rates in human populations are significantly higher in areas of high *A. terrestris* abundance (Viel et al., 1999), an example where high abundance in the primary intermediate host contributes to infections rates in a secondary (dead-end) intermediate host. It's possible that *A. terrestris* plays a similar role with other parasites and other hosts, with high *A. terrestris* abundance contributing to higher infections rates and disease in other more vulnerable hosts.

Abundant *A. terrestris* may also act to reduce transmission rates of parasites that they are not competent hosts for; this may occur if, for example, high *A. terrestris* abundance diverts ticks away from competent hosts. An example of this is *Borrelia burgdorferi* (Lyme disease) prevalence in *Ixodes ricinus* ticks in eastern United States; here, *Peromyscus maniculatus* is the primary competent host, but *B. borgdorferi* prevalence in ticks is inversely correlated with the abundance of incompetent hosts, including chipmunks, grey squirrels, and white-tailed deer (Ostfeld and Keesing, 2000).

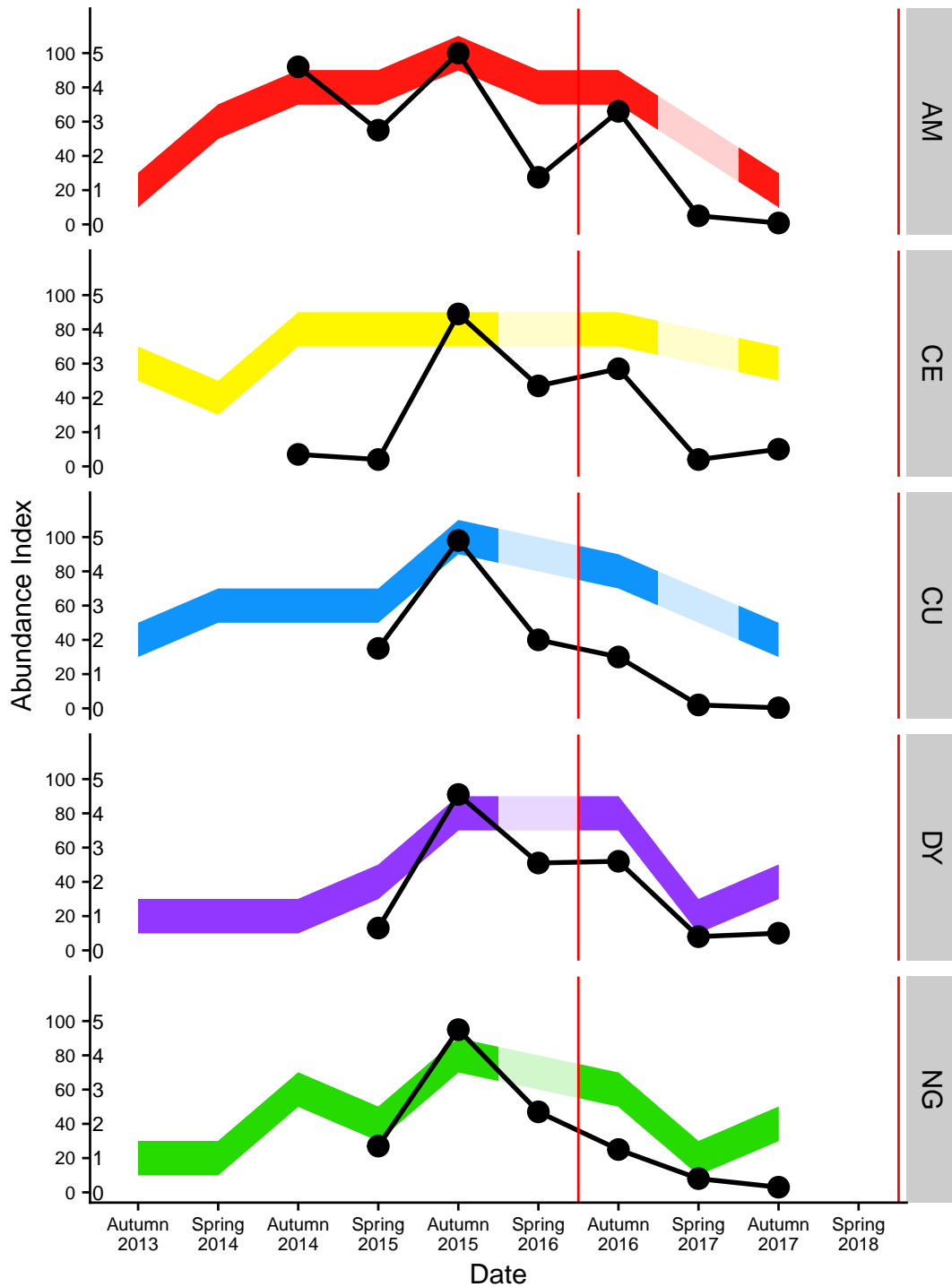
#### 5.3.4 Next Steps: 2016-2018

Our immediate next steps will be analyzing the samples collected after spring 2016 that were not processed in time for inclusion in this thesis. As shown in Figure 5.1, local and commune-level abundances of *A. terrestris* tend to decline after spring 2016. How bacterial communities change over time, and if those changes differ between locations will be the focus of our future investigations.

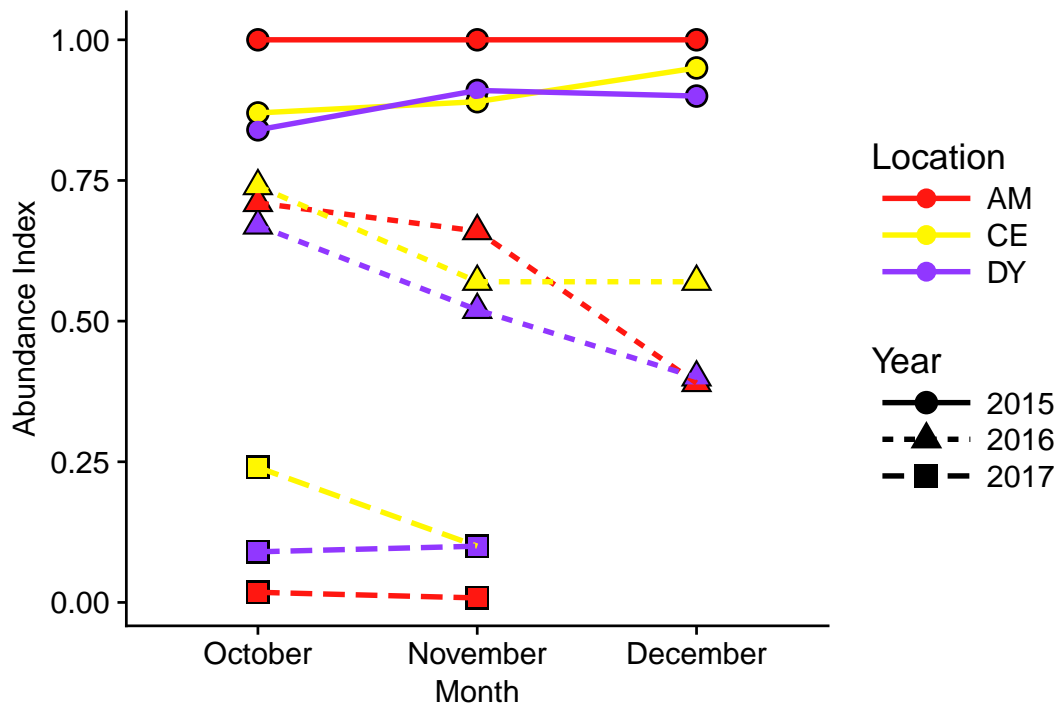
Does mean infracommunity richness decline with declining abundance, or does it remain high as abundance declines (delayed density dependence)? Cerqueira et al. (2006) reported that helminth infracommunity richness exhibited delayed density dependence in *A. terrestris*, with the highest richness documented in declining populations. Does component community similarly remain uncorrelated with geographical distance as populations decline, or does spatial structure emerge as abundance (and presumably connectivity) decline? Will we find more of the indicators identified in 2003-2005 in spleens collected from autumn 2016 and later? These will be some of the questions we hope to answer.

Krebs (2013) argued that short generation times in rodents warranted sampling frequencies on the order of 2-3 weeks to meaningfully capture population attributes, but in the





**Fig. 5.1.:** Abundance estimates for *A. terrestris* in our five communes in Franche-Comté, France, from autumn 2014 - spring 2018. Lines indicate changes in abundance indices estimated using walked line transects (longest transect, 1.2 km), while coloured bands indicate changes in abundance indices based on visual assessments conducted at the commune level. Pale sections of the bands indicate missing data. Sampling sessions in which animals were collected and dissected are indicated with solid circles; samples included in the analyses presented in this thesis are to the left of the vertical red lines; samples still to be analyzed are between the two red lines.



**Fig. 5.2.:** Local transect-based abundances of Arc-sous-Montenot (AM), Censeau (CE) and Doye (DY) in October, November and December of 2015, 2016, and 2017 (no data for December 2017 due to heavy snowfall). Animals were trapped and dissected each month following the protocol described in Chapter 2

analyses presented in this thesis, we've sampled population twice a year in spring and autumn, with the assumption that the time points that we've selected (early and late in the typical reproductive period) are meaningful in the context of parasite communities and host population dynamics. But we don't actually know if bacterial communities vary much over the short term, or how measures like infracommunity richness vary between the time points that we have sampled (spring and autumn). We think this is particularly important in the autumn, because during the peak phase, local *A. terrestris* exhibit seasonal oscillations with an over-winter decline that begins as early as October. Populations either recover to high density in the spring, in which case the population is still in the peak phase of the outbreak, or they fail to recover the following year, and the decline phase has occurred (Pascal and Boujard, 1987). How do parasite communities change over short periods when population abundances can fall rapidly?

To address this question, we have sampled three of our sites in October, November and December 2015-2017, with the intention of capturing these seasonal oscillations (see Figure 5.2) and characterizing short-term variation in bacterial communities. We wish to determine if there are significant changes in measures like infracommunity richness over these short time periods, and identify intrinsic and extrinsic factors at the individual and population level that predict such changes.

### 5.3.5 Identifying Bacterial Parasite Species Interactions

Given the diversity of parasites we have detected and those already documented in *A. terrestris*, we turn our attention now to analyzing patterns of parasite associations. One approach for exploring parasite associations is the concept of nestedness, in which infracommunities are nested subsets of the component community (i.e. rare taxa are found only in species-rich infracommunities, while common taxa are found in species-rich and species-poor infracommunities) (Poulin, 1996). This pattern is represented graphically in Table 5.2. Ulrich et al. (2009) describes a three-step process to nestedness analysis, in which 1) nestedness is quantified using a metric, 2) the metric is compared to an appropriate null model or randomization test to assess statistical significance, and 3) the mechanism generating the pattern of nestedness is inferred. Nestedness in infracommunities can be found in some systems (e.g. monogean ectoparasites of some tropical fish (Guégan and Huguény, 1994)) but is absent from many others (Poulin, 1996).

**Tab. 5.2.:** Presence-absence matrix for parasite taxa in hosts, organized to demonstrate the concept of nestedness. Rows (parasite taxa) and columns (hosts) are organized by their sums, with common parasites at the top and parasite-rich hosts to the left. Nestedness is then the concentration of presences in the upper left corner of the matrix. Adapted from Ulrich et al. (2009)

Parasite	Host								Sum
	A	C	F	D	G	E	B	H	
1	1	1	1	1	1	1	1	1	8
2	1	1	1	1	1	1	0	0	6
3	1	1	1	1	1	0	1	1	7
4	1	0	1	1	1	1	0	0	5
5	1	1	1	0	0	1	1	0	5
6	1	1	1	0	0	1	0	0	4
7	1	1	0	1	0	0	0	0	3
8	1	1	1	0	0	0	0	0	3
9	1	1	0	0	0	0	0	0	2
10	1	0	0	0	1	0	0	0	2
11	0	0	0	1	0	0	0	1	2
12	1	0	0	0	0	0	0	0	1
Sum	11	8	7	6	5	5	3	3	48

An alternative approach is to look for specific pairs or groups of parasites that are associated; that is, they co-occur in hosts more frequently than expected given a random distribution in the host population. This method may be more appropriate in instances where specific pathogens have been targeted for detection in the host, rather than the community as a whole, or when hypotheses concerning specific species pairs or groups are being tested. Vaumourin et al. (2014) compared generalized chi-square tests, networks, multinomial GLMs and their own screening approach for detecting significant associations between a set of blood parasites in a population of field voles (*Microtus*

*agrestis*) and found that *Bartonella doshiae* and *Babesia microti* co-occurrences were significantly under-represented (negatively associated), a finding also reported by Telfer et al. (2010) for this system using time-series data. However, these methods did not detect significant associations between *Anaplasma phagocytophilum*, *Bartonella* sp., and *Babesia microti*, which were detected by (Telfer et al., 2010) using longitudinal data.

In addition, the bacteria that we have found are not only taxonomically diverse, they are also ecologically diverse in the tissues they target, their propensity for causing disease, their transmission mechanisms, their host specificity, etc. (see appendices for details). Now that we have summarized bacterial parasite communities in a broad sense, further investigations into specific functional groups (e.g. haemotrophic bacteria that can compete for host red blood cells, or bacteria that interact indirectly through cross-protection; Bajer et al. 2014; Birtles et al. 2001) are warranted.

## 5.4 Further Questions

### 5.4.1 Spatial Scales

Future avenues that warrant exploration include questions of scale. We attempted to trap animals that inhabited separate tunnel systems, and when local abundance was low delineation of tunnel systems was clear and thus we were confident that we were sampling separate burrows. At high abundances, when transect indices exceeded 50 %, burrow systems were harder to delineate, and given that tunnels can exceed 100 m in total length and are often occupied by more than one animal (Airoidi, 1976b), we may have captured animals that were in fact sharing a tunnel system. Social organization can have important implications for disease transmission rates; Alcock (1989) suggested that sociality increases susceptibility to infection, but Cross et al. (2004) have argued that sociality can contain disease spread by reducing population mixing. Parasite communities, by extension, are also expected to be influenced by sociality; Bordes et al. (2013) reported a significant negative relationship between sociality and ectoparasite richness in rodents, with more social rodents hosting less-rich ectoparasite communities. Two possible explanations were presented for this trend: higher sociality in the host constituted a more homogeneous environment for the parasites, leading to increased competition between parasites and lower species richness, and increased sociality may have co-evolved with strategies to reduce parasitism or parasite transmission, such as auto- and allogrooming. *A. terrestris* tunnel occupancy (number, age and sex of occupants) can change with burrow size which in turn varies with season (Airoidi, 1976b), thus some of the patterns and changes in infracommunity richness that we observed may be due to very local conditions (i.e. tunnel co-inhabitants and changes therein), and this needs to be explored further.

From a larger perspective, we've sampled five locations that are within 20 km of each other, but *A. terrestris* population outbreaks are a regional phenomenon (the Jura plateaus) and also occur in neighbouring Switzerland. We currently have no measure of the regional or landscape bacterial parasite community, and these species pools are likely to play a role in the local patterns we have observed (Guégan et al., 2005).

## 5.4.2 Temporal Scales

We've discussed several methods for identifying parasite associations from cross-sectional data, but understanding how parasite infection influences things like the probability of infection by another agent, host reproduction, or host survival, etc. requires longitudinal data in which animals are sampled but released (Telfer et al., 2010; Telfer et al., 2005; Burthe et al., 2008). In addition, longitudinal sampling can provide insights into infection duration, which can vary considerably between parasite species, host individuals, and be influenced by co-infection (e.g. Birtles et al., 2001).

Longitudinal data may also be important in understanding the conditions under which commensal microparasites transition to pathogenic agents, *sensu* the pathobiome concept, in which a pathogenic agent is integrated and understood within its biotic environment (Vayssier-Taussat et al., 2014). One of the key aspects of the pathobiome concept is the recognition that species that may normally be benign or beneficial components of the host microflora can become pathogenic under certain conditions; an example of such a conversion relevant to our system can be found in some members of *Mycoplasma* sp., which can be isolated from the respiratory tract of clinically normal animals but can become pathogenic and exacerbate already-established infections (Whithear, 2001). Identifying under what exact conditions mycoplasmas and other commensals become pathogenic would be best approached using longitudinal sampling.

## 5.4.3 Consequences of Parasitism

The previous section focused on questions of temporal and spatial scale, but another important area of future research is elucidating the mechanisms underlying the patterns we have observed, and determining if parasite community structure has consequences for *A. terrestris* individuals and populations. We anticipate that these mechanisms and consequences are complex, and likely not independent; Beldomenico et al. (2008a) has reported that *Microtus agrestis* in poor condition (as measured by lymphocyte and red blood cell counts) were more likely to exhibit elevated immunological indicators of infection (monocytosis or neutrophilia) when sampled four weeks later, and that animals with high infection indicators were more likely to later develop signs of low body condition. In addition, Beldomenico et al. (2009) reported higher infection intensities

of *Trypanosoma microti* developing in *Microtus agrestis* with pre-existing poor body condition, which raises the possibility of "superspreaders", or individuals that contribute disproportionately more than the average infected host to the transmission of parasites (Lloyd-Smith et al., 2005).

These patterns of "vicious circles" in which poor condition predisposes individuals to infection and infection predisposes to declining body condition, and the heterogeneity in susceptibility to entering this circle in the host population, may be important in shaping infracommunity structure in *A. terrestris*. Charbonnel et al. (2008b) reported increased fecal corticosterone metabolite concentrations from *A. terrestris* collected during the decline phase compared to animals from the peak (pre-decline) phase; this measure of physiological stress was negatively correlated with body condition, which in this case was estimated using the residuals of log-transformed body mass over log-transformed age. Immune function also appeared to be impaired in decline-phase voles, especially in females that had not yet reproduced, which could be a contributing factor in the decline. Thus, decline-phase *A. terrestris* is characterized by a suite of interacting factors (poor body condition, elevated stress and poor immunocompetence) that are expected to influence susceptibility to infection.

Other consequences to explore include parasite communities and direct mortality, reproduction, and predation risk. Individual parasite species can cause mortality in *A. terrestris* (e.g. experimental infections of *Mycobacterium microti* in *A. terrestris* are ultimately fatal; Jespersen, 1974), but the effect of coinfections is currently unknown. Helminths exacerbate *Mycobacterium bovis* infections in African buffalo (*Syncerus caffer*) to the point where high mortality in co-infected animals contributes to a negative correlation between helminth infection and tuberculosis at the population level (Jolles et al., 2008), thus coinfections may be an important source of mortality and a contributor to patterns of species associations in *A. terrestris*.

Parasitism may also cause mortality indirectly by increasing the host's risk of predation, especially in parasites that have indirect life cycles and rely on a predator as definitive host; for example, *Toxoplasma gondii* infection in rat intermediate host alters anti-predation behavioural traits specifically to increase chances of predation by cat definitive hosts (Lamberton et al., 2008). In host-parasite systems with direct parasite life cycles, the effect of parasitism on predation risk varies from system: Haukisalmi et al. (1994) failed to find any relationship between helminth burden and risk of predation by owls in *Microtus agrestis*, Sunde et al. (2012) found that *Apodemus flavicollis* taken by tawny owls had lower prevalences of the intestinal parasite *Helimosomoides* sp. than animals that were live trapped from the same population, and Isomursu et al. (2008) found that grouse hunted with a dog were more heavily parasitized with cestodes than grouse hunted without a dog, suggesting that cestode parasitism increased the vulnerability of

grouse to predation by wild predators. Whether infection by bacterial parasites alters predation risk should be explored in our system.

Parasites can also influence immigration rates by either increasing or decreasing propensity to disperse and/or modifying dispersal distance; in roe deer (*Capreolus capreolus*) in south-western France, higher nematode burden coupled with lower body mass was found to be correlated with a reduced propensity to disperse (Debeffe et al., 2014). Parasites may play a hitherto unknown role in *A. terrestris* dispersal and the observed patterns in population isolation and connectivity observed over the course of the outbreak.

#### 5.4.4 Final Note

We have come a long way since Elton first suggested epidemic disease played a role in European rodent cycles, and while our understanding of infection, disease and population ecology has developed considerably, an emphasis on single parasites and delayed density dependence has persisted in the literature until quite recently. We have argued that to consider a single parasite species in isolation is to miss the complex picture that is the parasite community, and that parasite communities themselves may be a more meaningful scale to consider in studies of host-parasite dynamics. We have demonstrated that bacterial parasite communities vary between individuals and populations, and through time and space, and in presenting these patterns we have laid the groundwork for further investigations into bacterial parasite communities in *A. terrestris* in Franche-Comté.

Supplementary Material for Villette  
et al. (2017) - Short Report -  
Consequences of organ choice in  
describing bacterial pathogen  
assemblages in a rodent population

- Supplementary Material 1 - Sequencing Methods - page 100
- Supplementary Material 2 - OTU Accumulation Curves - page 108
- Supplementary Material 3 - Case Study - page 110
- Supplementary Material 4 - Individual Effects - page 113



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3 population.

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5 **Supplementary Material 1 - Sequencing methods**

6 PCR amplification, indexing, multiplexing and demultiplexing followed Kozich et al. [1]  
7 with modifications detailed by Galan et al. [2]. Negative controls and general procedures for  
8 removing biases and ensuring the quality of the data for later interpretation followed the  
9 guidelines in Table 1 of Galan et al. [2]; we used *Mycoplasma putrefaciens*, *Mycoplasma*  
10 *mycoides*, *Borrelia burgdorferi*, and *Bartonella taylorii*, for positive PCR amplification controls.

11 We used primers 16S-V4F/16S-V4R dual-indexed with 8bp-indices (i5 and i7) and  
12 Nextera Illumina adapters to perform two PCR replicates for each sample in 5 µL of 2 ×  
13 Multiplex PCR Kit (Qiagen), 4 µL of combined forward and reverse primers (2.5 µM) and 2 µL  
14 of DNA extract. The PCR program consisted of an initial denaturation step at 95°C for 15  
15 minutes, followed by 40 cycles of denaturation at 95°C for 20s, annealing at 55°C for 15s, and  
16 elongation at 72°C for 5 minutes, followed by a final extension step at 72°C for 10 minutes. PCR  
17 amplification was verified by electrophoresis in a 1.5% agarose gel. Pooled amplicons were size-  
18 selected by excision after gel electrophoresis with 1.25% agarose gel in 1% TAE buffer, and were  
19 purified using the NucleoSpin Gel clean-up kit (Machery-Nagel Sarl, France). We performed  
20 quantitative PCR with the KAPA library quantification kit (KAPA Biosystems) as per [2] to  
21 quantify the DNA of the final library, and then loaded the library on the Illumina MiSeq flow cell  
22 using a 500 cycle reagent cartridge and 2 x 251bp paired-end sequencing, which yielded high-

23 quality sequences through the reading of each nucleotide of the V4 fragments twice after the  
24 assembly of reads 1 and reads 2. The raw sequence reads are available in FASTQ format in the  
25 Dryad Digital Repository.

26         Sequence analysis and taxonomic classification were performed using the mothur  
27 program package [3] and following the standard operation procedure of Patrick D. Schloss [1].  
28 We used the Silva SSU Reference database v119 [4] for taxonomic assignment. To discard false  
29 negatives introduced by biases in the procedure described above, we filtered the read-abundance  
30 table generated by mothur for each PCR product and each OTU following Galan et al. (2016).  
31 We used negative controls for both DNA extraction (1 well per 96-well extraction plate) and PCR  
32 amplification (1 well per 96-well PCR plate) to estimate cross-contamination rates for individual  
33 OTUs, and we used a false-assignment rate of 0.02% to filter read abundances. To account for  
34 potential false-positives, we considered a sample positive for a given OTU only if that OTU was  
35 present in both PCR replicates after the filtering steps described above were applied. Our  
36 mycoplasma positive controls were assigned to Entomoplasmataceae rather than  
37 Mycoplasmataceae due to a frequent taxonomic error reflected in most databases, including Silva  
38 (23).

39         We classified OTUs as potentially pathogenic using the 2nd edition of Bergey's Manual  
40 of Systematic Bacteriology, Volumes 1 to 5 [5–9], Pathogenesis of Bacterial Infections in  
41 Animals [10] and a general search of the literature. If we found documentation implicating a  
42 genus in an infection or disease in mammals, all OTUs in that genus were designated as  
43 potentially pathogenic. If an OTU could not be identified to the genus level, and was assigned to a  
44 clade that has no known pathogenic members, these OTUs were discarded from the analysis as  
45 contaminants, commensals, or rare taxa of unknown function. For the remaining unclassified

46 OTUs that were assigned to clades that contain pathogenic members, we compared individual  
47 sequences from these OTUs to the NCBI's GenBank database using BLAST [11], and potential  
48 pathogenicity status was assigned when a sequence matched a known pathogen, or matched a  
49 sequence implicated in an animal infection or disease.

50         Summaries of the number of reads produced and retained during the analysis are provided  
51 in Supplementary Table 1. The total number of reads retained in each sample after data filtering  
52 procedures were performed are summarized in Supplementary Table 2. A brief summary of the  
53 OTUs removed from the data set during data filtering procedures is provided in Supplementary  
54 Table 3.

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- 85
- 86

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Supplementary Table 1: Summary of the number of reads produced and retained by our sequencing protocol and post-sequencing data filtering and analysis procedures.

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Total number of reads before filtering	1154658
Total number of reads removed after filtering procedures	897725 (77.7%)
Total number of reads retained after filtering procedures	256933 (22.3%)

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87

88

Supplementary Table 2. Read totals for each sample after data filtering.

Location	Animal	Heart	Liver	Lung	Kidneys	Spleen
A	1	7658	0	1784	2042	622
A	2	44	40	0	1148	22
A	3	2062	0	0	11	17277
A	4	9974	3160	5008	1805	1872
A	5	24929	10550	14762	8640	13649
A	6	1296	1396	955	763	40888
A	7	0	0	0	8	0
A	8	4403	343	335	6144	653
A	9	35	0	20950	0	0
C	10	44	105	0	121	698
C	11	279	103	2769	20500	1132
C	12	2114	3031	6057	10216	31
C	13	4207	0	259	10	29

89

90

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Supplementary Table 3: The number of OTUs per bacterial phylum

that were removed from the data set during data filtering procedures.

---

Phylum	Number of OTUs
Acidobacteria	45
Actinobacteria	131
Bacteroidetes	302
Candidate_division_OD1	2
Candidate_division_WS3	2
Chlamydiae	2
Chlorobi	1
Chloroflexi	10
Cyanobacteria	18
Deferribacteres	3
Deinococcus-Thermus	2
Elusimicrobia	3
Firmicutes	474
Gemmatimonadetes	7
Nitrospirae	1
Planctomycetes	42
Proteobacteria	249
SHA-109	1
Spirochaetae	1
Tenericutes	25
TM6	5
unclassified	100
Verrucomicrobia	30
WCHB1-60	1

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1 *Epidemiology and Infection*

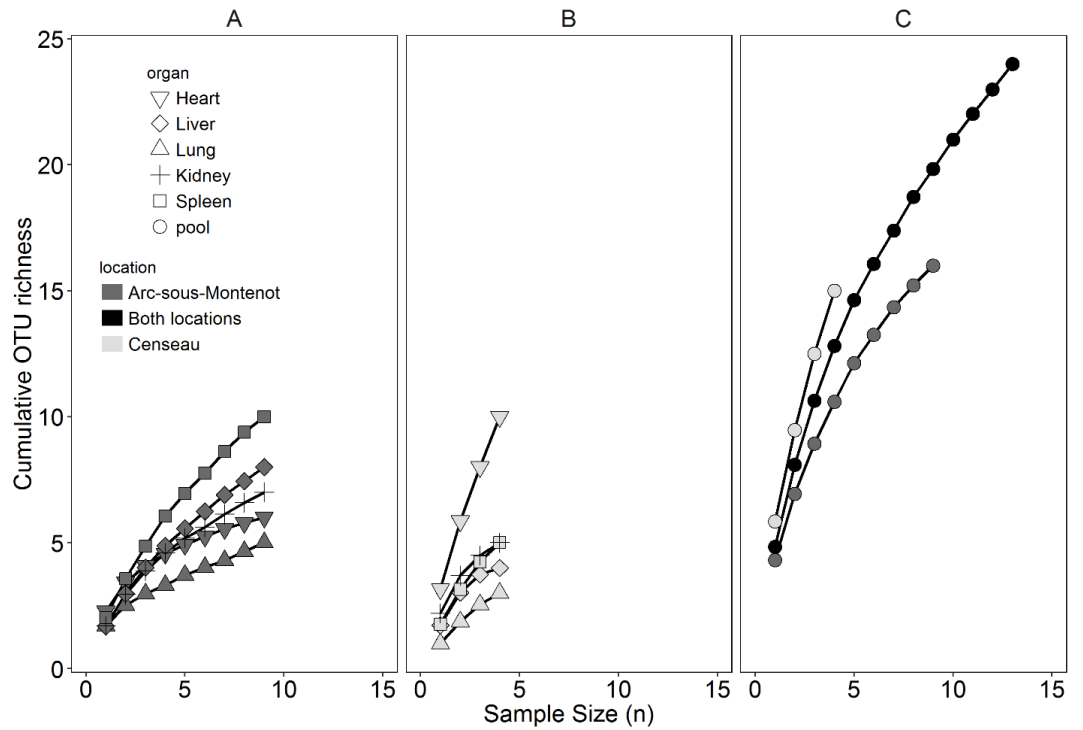
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3 population.

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5 **Supplementary Material 2 - OTU accumulation curves**

6 We constructed OTU accumulation curves for each organ and pooled organs within  
7 locations, as well as pooled organs across locations to assess the effects of increasing sample size  
8 on the number of OTUs detected.

9 OTU accumulation analysis of each organ indicates that with the exception of livers from  
10 Censeau, increasing the number of animals sampled increases the number of OTUs detected  
11 (Supplementary Figure S1). OTU detection is maximized within location when all organs are  
12 pooled (15 OTUs and 16 OTUs in Censeau and Arc-sous-Montenot, respectively), and  
13 maximized across locations when all organs from both locations are pooled (24 OTUs).



14

15 Supplementary Figure S1. Bootstrapped OTU accumulation curves (999 iterations) of the number  
 16 of potentially pathogenic bacterial operational taxonomic units (OTUs) detected in the heart,  
 17 lung, liver, spleen, and kidneys of *Arvicola terrestris* collected from A) the commune of Arc-  
 18 sous-Montenot, France, B) the commune of Censeau, France. OTU accumulation curves for  
 19 when organs are pooled within animals (pool) are also shown for both communes individually  
 20 and together (C).

2 Consequences of organ choice in describing bacterial pathogen assemblages in a rodent  
3 population.

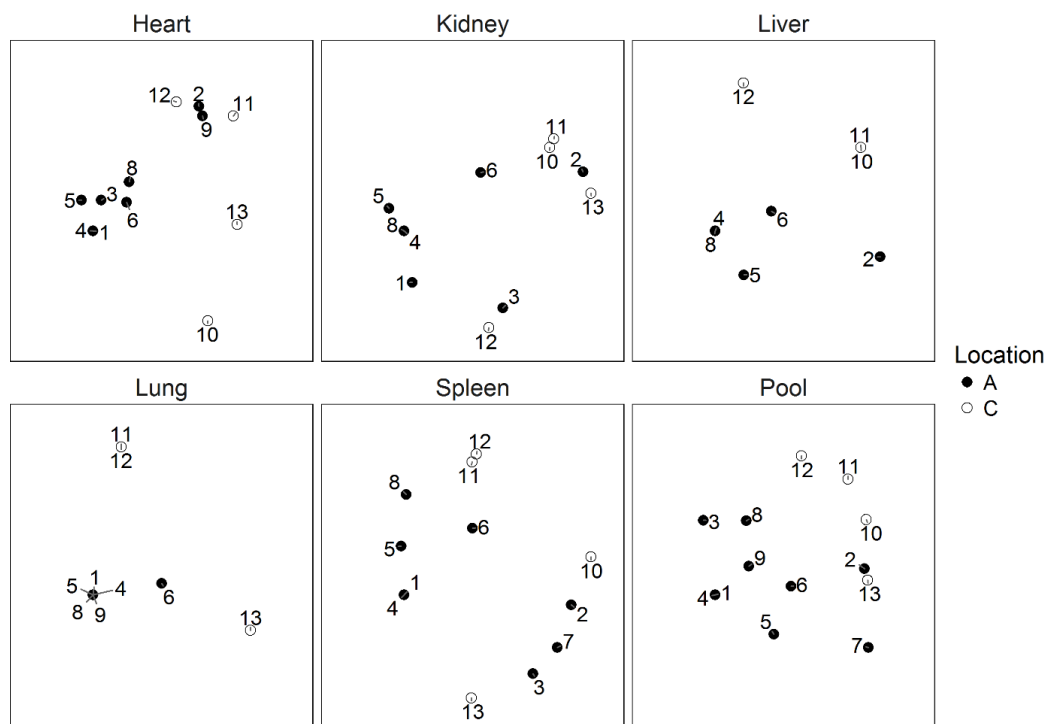
4 P. Villette, E. Afonso, G. Couval, A. Levret, M. Galan, C. Tatar, J.F. Cosson, P. Giraudoux

5 **Supplementary Material 3 - Case study**

6 We used a case study to explore the consequences of organ choice in detecting host-  
7 population-level differences in bacterial assemblages. We used pooled bacterial assemblages for  
8 each animal to test for population-level differences, and then conducted the same test with each  
9 organ to determine if all organs generate the same conclusion. We predicted that host-population-  
10 level differences in bacterial assemblages would be more easily detected in the liver and spleen  
11 because these two organs are likely to host the greatest bacterial richness, and that lower bacterial  
12 richness in the other organs would make detecting population-level differences, if they existed,  
13 more challenging.

14 We used nonmetric multidimensional scaling ordination (NMDS) to visualize Jaccard  
15 dissimilarities between individual organ bacterial assemblages and the bacterial assemblages of  
16 each animal when organs were pooled together. We conducted PERMANOVAs with each organ  
17 individually with a Bonferonni correction of  $\alpha=0.01$  to determine if each organ's OTU  
18 community differed between sites. We then pooled organ data within animals to determine if  
19 using all organs changed the PERMANOVA results (ie. whether OTU assemblages differed  
20 between Arc-sous-Montenot and Censeau).

21 NMDS ordination of Jaccard dissimilarity indices shows that when organ assemblages are  
 22 pooled, Arc-sous-Montenot and Censeau animals form distinct clusters (Supplementary Figure  
 23 2). Clustering within organ types is less clear, but the PERMANOVA analyses detected location  
 24 effects when all organs are pooled together, and when the kidney was considered alone, but not  
 25 when the spleen, liver, lung, or heart assemblages are viewed alone (Supplementary Table 4).



26

27 Supplementary Figure S1. Jaccard dissimilarity-based NMDS ordination of potentially pathogenic  
 28 bacterial assemblages in the heart, liver, lungs, kidneys, spleen, and pooled organs of 13 male  
 29 *Arvicola terrestris* collected in November 2014 in the communes of Arc-sous-Montenot (A) and  
 30 Censeau (C) in Franche-Comté, France. Individual animals are indicated by number; in instances  
 31 where points overlap, numbers are shown next to their respective points (e.g. animals 1,4,5,8 and  
 32 9 had identical lung assemblages and therefore map to the same point in the lung panel).

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Supplementary Table S4. PERMANOVA results for location effects for each organ individually, and for organs pooled within animals. A bonferonni correction of  $\alpha=0.01$  was applied to the individual organ PERMANOVAs.

Organ	F	df	R2	p	significant
Heart	2.94	1,10	0.23	0.045	no
Liver	3.53	1,6	0.37	0.02	no
Lung	10.29	1,7	0.60	0.011	no
Spleen	1.77	1,10	0.15	0.06	no
Kidney	3.20	1,9	0.26	0.009	yes
Pool	2.92	1,11	0.21	0.008	yes

---

1 *Epidemiology and Infection*

2 Consequences of organ choice in describing bacterial pathogen assemblages in a rodent  
3 population.

4 P. Villette, E. Afonso, G. Couval, A. Levret, M. Galan, C. Tatar, J.F. Cosson, P. Giraudoux

5 **Supplementary Material 4 – Individual effects**

6 We conducted a PERMANOVA analysis to determine if bacterial assemblages are influenced by  
7 individual identity; assemblages were permuted within organs but between animals, and locations  
8 were considered separately. In both Arc-sous-Montenot and Censeau, we found that assemblages  
9 are not assorted randomly, but differ between animals (PERMANOVA, pseudo- $F_{8,33}=3.47$ ,  
10  $p=0.001$  in Arc-sous-Montenot and pseudo- $F_{3,17}=1.93$ ,  $p=0.002$  in Censeau).



## Parasitic Bacteria in *Arvicola terrestris*

We present here a brief summary of what information is available regarding the ecology, transmission, host specificity and pathogenicity of the bacterial species we detected. Several of the bacteria we identified are widespread within the animal kingdom, and have been documented in other rodent species or other populations of *A. terrestris*, thus their presence in *A. terrestris* is not surprising.

### B.1 *Bartonella*

*Bartonella* sp. are small obligate intracellular parasites infecting the endothelial cells and erythrocytes of hosts (Jacomo et al., 2002). They can infect a variety of animals including domestic and wild canids (Breitschwerdt et al., 1995; Beldomenico et al., 2005), domestic cats (Heller et al., 1997), and rabbits (Heller et al., 1999), but the majority of strains described to date have been isolated from rodents (Gutiérrez et al., 2015).

*Bartonella* colonizes the spleen and liver within the first week post infection (Deng et al., 2012), before invading erythrocytes where they replicate to produce up to 8 daughter cells; infected erythrocytes may then persist for at least several weeks, with subsequent waves of infection of new erythrocytes every few days (Schülein et al., 2001). Infected erythrocytes may then be ingested by blood-sucking ectoparasites, who transmit the bacteria to other hosts via their feces (see below).

A degree of host specificity has been documented in rodent-borne strains of *Bartonella*; Kosoy et al. (2000) were able to experimentally induce bacteremia in cotton rats (*Sigmodon hispidus*) and white footed mice (*Peromyscus leucopus*) only with *Bartonella* strains obtained from the same rodent species or a close relative.

Transmission is primarily by vector; bacteria enter the host via infected arthropod feces being scratched into the skin (Chomel et al., 2009). In rodents, fleas are thought to be the dominant vector of transmission (Bown et al., 2004; Chomel et al., 2009; Marie et al., 2006); lice, ticks, mites, and biting flies are also associated with *Bartonella* (Billeter et al., 2008; Byam and Lloyd, 1920; Halos et al., 2004; Kim et al., 2005). Vertical transmission



has been observed in some species (e.g. *Peromyscus leucopus* and *Sigmodon hispidus*, Kosoy et al., 1998) but not others (e.g. *Myodes glareolus*, Bown et al., 2004), and in general is unlikely to play a significant role in *Bartonella* dynamics in wild populations (Gutiérrez et al., 2015). Transmission of *Bartonella quintana* from cats to humans by cat saliva has also been suggested but is not well understood (Breitschwerdt et al., 2007).

*Bartonella* in humans can cause serious diseases including cat-scratch disease, trench fever, and Carrion's disease/Oroyo fever (reviewed by Maguiña et al., 2009), but infections in animal reservoir hosts are generally asymptomatic or sub-clinical and don't lead to disease (Pulliainen and Dehio, 2012). Infection in non-reservoir species can lead to serious disease, including changes in reproductive success in mice (Boulouis et al., 2001).

*Bartonella* sp. has been isolated from rodents and their arthropod ecto-parasites all over the world, see Buffet et al. (2013) and Gutiérrez et al. (2015) for extensive reviews. We list reports of *Bartonella* infection in other *Arvicola* sp. populations in Table B.1, as well as arthropods that have been identified as potential vectors of *Bartonella*.

**Tab. B.1.:** *Bartonella* that have been isolated from *A. terrestris*, and vectors that are associated with the *Bartonella* species (not necessarily with *A. terrestris*)

Reference	<i>Bartonella</i> species	Vector
Oliver et al. (2009) (Scotland)	<i>B. doshiae</i>	<i>Polygenis gwyni</i> , <i>Ixodes turdus</i> (Kim et al., 2005; Buffet et al., 2013)
	<i>B. birtlessi</i>	<i>Ctenophthalmus nobilis nobilis</i> (Bown et al., 2004)
	<i>B. grahamii</i>	
Gelling et al. (2012) (UK)	<i>B. taylorii</i>	<i>Polyplax spinulosa</i> , <i>Xenopsylla cheopis</i> , <i>Ctenophthalmus nobilis nobilis</i> (Bown et al., 2004; Buffet et al., 2013)
	<i>Bartonella</i> sp.	

## B.2 *Bordetella*

*Bordetella* sp. inhabits the respiratory tracts of mammalian and avian hosts, and is spread by direct contact or aerosol (Register and Harvill, 2010). Infection can be clinically invisible or can lead to acute respiratory disease characterized by high morbidity and low mortality.

Soveri et al. (2000) isolated *Bordetella bronchiseptica* from 34% of *Microtus agrestis* collected during a winter decline, and observed two deaths due to *Bordetella* infection in 16 animals taken to the lab for experiments. *B. bronchiseptica* has also been isolated from the mountain vole *Microtus montanus* (Jenson and Duncan, 1980). Forbes et al.

(2015) reported that *B. bronchiseptica* infections could limit population abundance in food-supplemented populations of *Microtus agrestis*, likely due to congregation and increased transmission around feeding stations. *B. bronchiseptica* has been isolated from a variety of other animals including pigs, dogs, cats, guinea pigs, and rabbits (Brockmeier et al., 2008; Register and Harvill, 2010).

### B.3 *Brucella*

*Brucella* are obligate intracellular parasites of mammalian hosts (Roop et al., 2003) that localize in phagocytes and specialized epithelial cells (Olsen et al., 2010). Strains have been isolated from a range of hosts, particularly livestock (e.g. *B. abortus* in cattle, *B. melitensis* in goats, *B. suis* in swine, Thorne, 2001) where they can cause severe economic losses through abortions, infertility and weak offspring (Thorne, 2001; Olsen et al., 2010).

Strains have also been isolated from a number of wildlife species, including *B. microti* and *B. neotomae* from rodents. *B. microti* has been isolated from clinical samples of diseased *Microtus arvalis* in the Czech Republic (Scholz et al., 2008b) and red foxes from Austria (Al Dahouk et al., 2011). Unspecified *Brucella* strains have also been isolated from *Myodes glareolus* and *Apodemus* spp. in Germany (Hammerl et al., 2017). *B. neotoma* was isolated from wood rats (*Neotoma lepida*; Stoenner and Lackman, 1957). *Brucella microti* has also been isolated from soil (Scholz et al., 2008a), thus *Brucella* sp. doesn't strictly meet our criteria for inclusion in the analysis (survival and reproduction outside of a host is apparently not minimal), and further work to identify the strain of *Brucella* we have detected here is warranted.

As mentioned above, the first isolation of *Brucella microti* was from clinical samples taken from *M. arvalis* collected during an epizootic characterized by edematous extremities, skin lesions, enlarged lymph nodes and granulomas (Hubálek et al., 2007). In experimental infections, high doses of *B. microti* can be fatal for lab mice (Bagüés et al., 2010).

Transmission of *Brucella* sp. can vary from strain to strain: *B. abortus* and *B. melitensis* are considered virulent strains of *Brucella* and are transmitted by fluids and tissues associated with birth or abortions (Godfroid et al., 2004). Transmission of low pathogenic strains (*B. ovis* and *B. canis*) is through contact with mucous membranes, urine, or milk; venereal transmission in domestics is an important route of infection for these strains, as well as *B. suis* (Godfroid et al., 2004). Soil may be an important source for *B. microti* (see above). Upon entering the host, the bacteria invade macrophages, where they can persist and maintain chronic infection (Roop et al., 2003).

## B.4 *Candidatus Neoehrlichia*

*Candidatus Neoehrlichia mikurensis* is a group of bacteria within the Anaplasmataceae that was first isolated from ticks (*Ixodes ricinus*) in Europe (Alekseev et al., 2001; Schouls et al., 1999) and rats (*Rattus rattus*) in Asia (Pan et al., 2003). Identified as *Candidatus Ehrlichia* or *Ehrlichia*-like, Kawahara et al. (2004) demonstrated that these strains formed a related clade, which they designated *Candidatus Neoehrlichia mikurensis*.

As implied above, these bacteria are vector-borne, and do not appear to be transmitted transovarially within the tick host (Jahfari et al., 2012), thus they require a reservoir host. In Europe, rodents appear to be the primary reservoir; *Candidatus N. mikurensis* has been isolated from bank vole spleens *Myodes glareolus* in France at low prevalences (5 of 276 animals collected; Vayssier-Taussat et al., 2012), and it has also been isolated from the blood of bank voles (*M. glareolus*), field voles (*M. agrestis*), wood mice (*Apodemus sylvaticus*) and yellow-necked mice (*A. flavicollis*) in Sweden (Andersson and Råberg, 2011). In these cases, the strains isolated from the rodents matched strains isolated from human infections. Tissues from three *Arvicola* sp. individuals collected in Germany were examined via PCR for *Candidatus N. mikurensis* and were found to be negative for this bacterium (Silaghi et al., 2012); to our knowledge this is the first report of *Candidatus Neoehrlichia* in *A. terrestris*.

Within the rodent host, these bacteria localize in the spleen, where they infect endothelial cells of the venous sinuses (Kawahara et al., 2004). Clinical manifestations of disease in rodents infected by *Candidatus N. mikurensis* were not reported in the studies mentioned above, but infections in humans can result in severe febrile illness (Loewenich et al., 2010).

## B.5 *Filobacterium*

*Filobacterium* sp., formerly labelled as cilia-associated respiratory (CAR) bacillus, was one of several aetiological agents causing chronic respiratory diseases in experimental animal facilities prior to the introduction of barrier systems in the 1980s (Ike et al., 2016). Chronic respiratory disease in infected lab rodents is characterized by bronchitis and bronchiolitis, a build-up of mucous and pus in the lungs, and the infiltration of leukocytes into the lamina propria, but in other animals infection by CAR may not induce clinical symptoms (Bergottini et al., 2005).

Experiments with mice suggest that transmission is primarily by direct contact between animals as opposed to airborne transmission, and though infection with mice-derived

CAR elicited seroconversion in rabbits and guinea pigs, it didn't lead to clinical manifestations in these animals (Matsushita et al., 1989).

Strains have been isolated from laboratory populations of rats (Itoh et al., 1987), mice (Jacoby and Lindsey, 1998), and rabbits (Cundiff et al., 1994), domestic pigs (Nietfeld et al., 1995), calves (Nietfeld et al., 1999), and goats (Fernández et al., 1996), as well as wild rats in the United States (MacKenzie et al., 1981; Brogden et al., 1993) and wild red deer, chamois and roe deer in Northern Italy (Bergottini et al., 2005). To our knowledge, this is the first reporting of *Filobacterium* sp. in a wild rodent in Europe.

## B.6 *Leptospira*

Leptospire are bacteria of the genus *Leptospira*; this group includes saprophytic species which are typically found in water and soil, and animal pathogens (Krieg et al., 2010). 10 pathogenic species have been identified to date, but these can also be grouped based on antigen reactivity into serovars nested within serogroups - members of a given serovar can be from different species (i.e. phylogeny and serovars don't align; Krieg et al., 2010). DNA sequencing of the 16S rRNA gene is sufficient to identify species, but serovar identification requires either variable number tandem repeats (VNTR) or multispacer sequence typing (MST), or microscopic agglutination tests (Adler and de la Peña Moctezuma, 2010).

Leptospire are ubiquitous globally, infecting animals on all continents except Antarctica (Ellis, 2015). In France, *Leptospira* sp. has been documented in a wide variety of mammals, including foxes, badgers, weasels, hares, squirrels and roe deer (Ayrat et al., 2016). *Leptospira* sp. has been detected in *Arvicola* sp. in the United Kingdom (Gelling et al., 2012) and Germany (Mayer-Scholl et al., 2014; Obiegala et al., 2016). Whether Franc-Comtois *A. terrestris* is a maintenance host for a given strain of *Leptospira* is unknown.

Leptospire concentrate in the kidneys after infection where they multiply and are shed in the urine (Faine et al., 1999); in chronic infections, they may be shed in the urine for months after initial infection (Athanasio et al., 2008). Contact with infected urine is the primary mode of transmission, but vertical transmission may also occur (Ellis, 2015). Chronically infected maintenance hosts are often asymptomatic (Athanasio et al., 2008), but when disease does manifest in domestics (pigs, cattle) it is often characterized by abortion, stillbirths and weak offspring with poor survival rates (Adler and de la Peña Moctezuma, 2010).

Some serovars appear to be maintained primarily by one species; for example serovar Hardjo is maintained in cattle globally, and pigs are associated with serovars Pomona and Australis (Ellis, 2015). In wildlife, this specificity is less clear; Ayrál et al. (2016) reported that 41 of the 42 European hedgehogs they found positive for *Leptospira* were infected with strains belonging to serovar group Australis, but carnivores hosted a variety of serovars.

## B.7 *Mycoplasma* and *Ureaplasma*

Mycoplasmas are small (300nm diameter) cell-wall-less bacteria of class Mollicutes; members include genera *Mycoplasma* sp., *Ureaplasma* sp., and *Alcholeplasma* (Browning et al., 2010). Their small genome size (as short as 580 kbp) renders them obligate parasites, dependent on hosts for the manufacture of essential compounds they cannot produce themselves (Baseman and Tully, 1997); with the exception of some mycoplasmas capable of forming biofilms, the majority do not survive outside of a host for long periods (Browning et al., 2010).

Mycoplasmas can be found parasitizing a wide variety of organisms including plants, mammals, birds, reptiles, fish, and arthropods; species infecting animals are predominantly *Mycoplasma* sp. (Whithear, 2001) but *Ureaplasma* sp. have been isolated from domestics and monkeys (Howard et al., 1978) and rats and mice are susceptible to experimental infection (Krieg et al., 2010). Mycoplasmas can be divided into two groups based on where they are typically found in the host: the hemotrophic mycoplasmas (or hemoplasmas) which typically parasitize host erythrocytes (Messick, 2004), and mucuous-membrane-infecting (MMI) mycoplasmas which are typically found parasitizing host respiratory and urogenital tracts (Neimark et al., 2001). Mycoplasmas were originally thought to be host specific, but the growing number of reports of mycoplasmas in "secondary" host species suggests that for many mycoplasmas host specificity is not very strong (Pitcher and Nicholas, 2005).

Transmission of the hemoplasmas *Mycoplasma haemomuris* and *Mycoplasma coccoides* among rodents is thought to be via an arthropod vector like the rat or mouse louse (*Polyplex spinulosa* and *P. serrata* respectively, Krieg et al., 2010), and transmission of *M. haemomuris* by the flea *Xenosylla cheopsis* has also been reported (Berkenkamp and Wescott, 1988). Transmission routes of many of the MMI mycoplasmas are currently unknown but likely depend on infection site (Krieg et al., 2010); mycoplasmas infecting the respiratory tract can be spread by aerosol or direct contact with mucuous membranes (Whithear, 2001), while those infecting the eye may be transmitted by flies (Marie-Pierre et al., 1999).

Pathogenicity of mycoplasmas varies considerably, and in general, infection appears to be the result of complex interactions between the mycoplasma, host, environmental conditions, and other parasites or pathogenic agents present (Whithear, 2001); a given mycoplasma can be isolated from both seriously diseased and clinically normal animals. When disease does occur, it varies in intensity from chronic low-grade infection to mortality. The most important clinical manifestations of MMI mycoplasmas are pneumonia, pleuropneumonia and other respiratory diseases (Whithear, 2001), but other infections include arthritis, generalized infection, septicemia, mastitis, and conjunctivitis (Browning et al., 2010).

Hemotrophic mycoplasmas in livestock are associated with low-grade anemia accompanied by declines in reproductive output and weight gain, milk or wool production, and increased risk of respiratory infections (Messick, 2004).

Wildlife populations can be severely impacted by mycoplasma-associated infections; infectious keratoconjunctivitis caused by *M. conjunctivae* is an important source of blindness-related mortality in ibex (*Capra ibex ibex*), mouflon (*Ovis orientalis musimon*), and chamois (*Rupicapra rupicapra rupicapra*; Sumithra et al., 2013). In bighorn sheep *Ovis canadensis canadensis*, *M. ovipneumoniae* was implicated in 47 % of cases of bronchopneumonia, a population-limiting disease for this species in western North America (Besser et al., 2008). However, there is no evidence of mycoplasma-induced disease in wild rodents except for *M. arthritidis* causing polyarthritis in rats; pathogenicity in the other rodent-infecting mycoplasmas listed in Table B.2 has only ever been documented in the laboratory.

The mycoplasmas that have been isolated in rodents are listed in Table B.2; some, like *M. pulmonis* are a serious problem in lab colonies, while others, like *M. microti*, were isolated from clinically normal animals and therefore their pathogenicity is unknown.

**Tab. B.2.:** Mycoplasmas that have been isolated from rodents, and any associated clinical manifestations.

Mycoplasma species	Host	Clinical Manifestations
<i>M. arthritidis</i>	<i>Rattus</i>	arthritis (Cole and Ward, 1979)
<i>M. cavipharyngis</i>	Guinea pigs	None documented (Hill, 1984)
<i>M. citelli</i>	Ground squirrels	None documented (Rose et al., 1978)
<i>M. coccoides</i>	<i>Mus</i> , Hamsters	anaemia (Thurston, 1954)
<i>M. collis</i>	Rodents	None documented (Hill, 1983a)
<i>M. cricetuli</i>	Hamsters	conjunctivitis (Hill, 1983b)
<i>M. haemomuris</i>	<i>Mus</i> , <i>Rattus</i>	anemia (opportunistic) (Tanaka et al., 1965)
<i>M. microti</i>	<i>Microtus pennsylvanicus</i>	None documented (Brown et al., 2001)
<i>M. muris</i>	<i>Mus</i>	None documented (McGarrity et al., 1983)
<i>M. oxoniensis</i>	hamsters	conjunctivitis (Hill, 1991)
<i>M. neurolyticum</i>	<i>Mus</i>	conjunctivitis (unconfirmed) (Nelson, 1950a; Nelson, 1950b)
<i>M. pulmonis</i>	<i>Mus</i> , <i>Rattus</i>	murine respiratory mycoplasmosis (Lindsey et al., 1985)

## B.8 *Mycobacterium*

The mycobacteria (the trivial name for *Mycobacterium* sp.) can be divided into a large group of free-living saprophytes found on dead and decaying matter, and a small group of obligate parasites (Grange, 1996). The obligate parasites include the *Mycobacterium tuberculosis* complex, the *Mycobacterium avium* complex, and some other pathogenic members. The *M. tuberculosis* complex includes, among others: *M. bovis* (bovine TB), a significant pathogen for domestics and some wildlife (Clifton-Hadley et al., 2001); *M. tuberculosis*, the causative agent of tuberculosis in humans; and *M. microti* (vole TB), which typically infects voles, mice and shrews (Wells and Robb-Smith, 1946), but has been isolated from a range of other animals including cats and pigs (Huitema and Jaartsveld, 1967), wild boar (Chiari et al., 2016), and humans (Niemann et al., 2000).

Transmission mechanisms of *M. microti* are not well understood, but in *Microtus agrestis* appear to involve the skin lesions that are a typical manifestation in advanced stages of the disease (Kipar et al., 2014). Cavanagh et al. (2004) reported a strong correlation between wounding and clinical signs of vole TB, which could suggest antagonist interactions are involved in transmission. Conversely, *M. bovis* is typically transmitted through inhalation or ingestion (Clifton-Hadley et al., 2001).

Advanced tuberculosis results in characteristic external lesions in voles (Wells and Oxon, 1937; Cavanagh et al., 2002), but prevalences estimated using only lesions are likely to be underestimates; Kipar et al. (2014) report that less than 50% of *Microtus agrestis* identified as being infected with *M. microti* via culture or histological examination exhibited external lesions. Tuberculosis in voles is progressive, and to date there is no evidence of animals recovering from lesions once they appear (Wells and Robb-Smith, 1946)

Experimental infections of *M. tuberculosis* and *M. bovis* in *A. terrestris* indicate that both species can cause disease in water voles, although the course of the infection tends to be chronic, with infected animals living for months after infection (Jespersen, 1974)

*Mycobacterium microti* was first documented in voles (*Microtus agrestis* and *Myodes glareolus*) in the United Kingdom in 1937 by Wells and Oxon (1937); prevalences in *M. agrestis* ranged from 9% to 31% depending on season and sampling location. More recent work on vole tuberculosis in *M. agrestis* populations at Kielder indicates that prevalence, assessed by the presence of characteristic lesions, exhibits a delayed density-dependent pattern, with a time-lag of 3 to 6 months (Cavanagh et al., 2004). We are unaware of any reports of *Mycobacterium* in wild *Arvicola* sp. populations.



## B.9 *Pasteurella* and *Actinobacillus*

*Pasteurella* and *Actinobacillus* are two of the six recognized genera in the *Pasteurellaceae* family, and obligate parasites of birds and mammals (Brenner et al., 2005a; Olsen et al., 2015). Members of this family can be divided into 21 clusters based on 16s rRNA sequences, of which 5 form named genera and the remaining are composed of what are considered misclassified members of the family (Olsen et al., 2015). One such cluster (the Rodent cluster) contains species that have been isolated from rodents, and includes *Pasteurella pneumotropica* and *Muribacter* [*Actinobacillus*] *muris*. Other members of this family that have been isolated from rodents include *P. multocida* (Quan et al., 1986), which is the most well-known member of this group due to its association with a range of economically important diseases, including hemorrhagic septicemia in cattle, atrophic rhinitis in pigs, and fowl cholera in birds (Boyce et al., 2010).

With the exception of *P. multocida*, the members of *Pasteurellaceae* are considered opportunistic pathogens, and can be found as benign members of the host respiratory, genital or alimentary tract microflora (Miller, 2001). For example, *Muribacter* [*Actinobacillus*] *muris* was first isolated from healthy lab mice (Bisgaard, 1986), but it can also be a significant pathogen in lab populations of mice (Anderson and May, 1979). Infections in wild mammals, when they do occur, can result in a wide range of clinical symptoms that can be organized into three categories: localized abscesses, respiratory disease, or septicemia (Miller, 2001). Transmission is via direct contact or the inhalation of aerosols; biting and scratching in rodents may also be important (Miller, 2001).

*P. pneumotropica* was isolated from a variety of South African rodents and was suspected of playing a role in population outbreaks and declines there (Shepherd et al., 1982).

## B.10 *Rickettsia*

Members of *Rickettsia* sp. are obligate intracellular parasites that typically utilize an invertebrate and vertebrate host to complete their life cycles (Brenner et al., 2005b). *Rickettsia* sp. has been reported in *Apodemus flavicollis*, *Myodes glareolus*, and *Microtus agrestis* in Germany (Schex et al., 2011), and *A. flavicollis*, *A. agrarius* and *M. glareolus* in Poland (Gajda et al., 2017).

Transmission to humans is by blood-sucking arthropods like ticks, but transmission dynamics between ticks and rodents is less clear; Pluta et al. (2010) reported detecting *Rickettsia* sp. in 31% of *Dermacentor* sp. ticks collected from *Microtus arvalis* and *A. terrestris* but none of the animals hosting infected ticks were themselves positive for *Rickettsia*. Burri et al. (2014) reported the *Rickettsia*-infected *Apodemus* sp. and *Myodes*

*glareolus* failed to transmit *Rickettsia* to uninfected ticks. The role of rodents as reservoir hosts is also not clear; *Rickettsia* sp. can be transmitted transovarially in ticks (Brenner et al., 2005b), and thus may not require a mammalian reservoir host to complete its life cycle (Gajda et al., 2017)

*Rickettsia* sp. are the causative agents of scrub fevers and typhus in humans, but little is known about their impact on rodent hosts.



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