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***Bartonella* spp. Infection in Edible Dormice and Their Fleas**  
Infekce bartonel u plchů velkých a jejich blech

MASTER'S THESIS

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## **Declaration**

Hereby I declare that I made this thesis independently, using the mentioned references. I have not submitted or presented any part of this thesis for any other degree or diploma.

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## Abstract

Bartonellosis is a group of emerging infectious diseases caused by facultatively intraerythrocytic bacteria transmitted between vertebrate hosts by arthropod vectors. Although only some *Bartonella* spp. are traditionally associated with human diseases, it was proposed that virtually any species can have zoonotic potential. The highest diversity of species was described from rodents acting as reservoirs with variable prevalence reaching up to 90%. Fleas associated with mammalian hosts play an important role in the *Bartonella* sp. lifecycle and are described to act not only as competent vectors but also as reservoirs for several species. The role of edible dormice (*Glis glis*), which often interacts with humans, and their fleas in the bartonellae transmission and lifecycle has so far been unknown; therefore, the prevalence and diversity of *Bartonella* spp. in edible dormice in the Czech Republic were studied. Complementary PCR analysis of four loci (*gltA*, *rpoB*, *ftsZ*, ITS), MALDI-TOF, and cultivation was performed to identify *Bartonella* sp. infections. The prevalence reaching 90% was found in dormice and their fleas. Altogether, four species were found and several genotypes of *B. grahamii*-like and yet undescribed *Bartonella* sp. were cultivated, and their morphology and growth requirements were determined. These results provide the first detailed information on the potential of the edible dormouse as a host and reservoir of potential zoonotic *Bartonella* species.

**Keywords:** *Bartonella*, edible dormice, *Glis glis*, fleas

## Abstrakt

Zástupci rodu *Bartonella* jsou fakultativně intraerycytární bakterie, které jsou mezi obratlovčími hostiteli přenášeny krevsajícími členovci. Bartonely mohou způsobovat lidská onemocnění, bartonelózy, které se řadí do skupiny tzv. emerging infectious diseases. Předpokládá se, že všechny druhy tohoto rodu mohou mít zoonotický potenciál. Nejvyšší diverzita bartonel byla popsána u hlodavců, přičemž detekovaná prevalence dosahuje až 90 %. Role plcha velkého (*Glis glis*) a s ním asociovaných blech v životním cyklu bartonel dosud není známá. Plši mohou sloužit jako hostitelé zoonotických patogenů, navíc epidemiologicky významní vzhledem k přítomnosti v lidských obydlích a jejich okolí. Blechy mohou sloužit nejen jako kompetentní přenašeči bartonel, ale i jako rezervoároví hostitelé. Cílem této práce je přispět k poznání prevalence a diverzity bartonel u plchů velkých v České republice. K identifikaci bartonelových infekcí byly použity metody PCR (*gltA*, *rpoB*, *ftsZ*, ITS), MALDI-TOF a kultivace. Detekovaná prevalence u plchů a jejich blech dosahovala až 90 %. Celkem byly detekovány čtyři druhy bartonel a dva (v několika genotypech) byly i úspěšně kultivovány (*B. grahamii*-like a dosud nepopsaný druh *Bartonella* sp.). Kultury byly použity k charakterizaci morfologie a optimálních růstových podmínek. Výsledky přinášejí podrobný přehled bartonel u plchů velkých v ČR a popisují možnou roli plchů jako hostitelů potenciálně zoonotických druhů rodu *Bartonella*.

**Klíčová slova:** *Bartonella*, plch velký, *Glis glis*, blechy

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## Abbreviations

BI: Bayesian inference

BTS: bacterial test standard

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

ELISA: enzyme-linked-immunosorbent-assay

EDTA: ethylenediaminetetraacetic acid

FCS: fetal calf serum

*ftsZ*: cell division protein

*gltA*: citrate synthase gene

*groEL*: chaperonin

HGT: horizontal gene transfer

HIV: human immunodeficiency virus

IFA: fluorescent antibody assay

ITS: internal transcribed spacer

LB: Luria-Bertani Medium

MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

ML: maximum likelihood

NCBI: National Centrum for Biotechnology Information

PBS: physiological saline solution

PCR: polymerase chain reaction

*ribC*: riboflavin synthetase

rpm: revolutions per minute

*rpoB*: RNA polymerase beta-subunit gene

SD: standard deviation

SDS: sodium dodecyl sulfate

SIMB: Schneider's insect medium-based medium

TEM: transmission electron microscopy

# 1 Introduction

Bartonellae are facultative intraerythrocytic bacteria with a wide variety of vertebrate hosts such as rodents, ungulates, carnivores, or humans (Brenner *et al.*, 1993; Breitschwerdt, 2017; Iannino *et al.*, 2018; Neves *et al.*, 2018; Sacristán *et al.*, 2021; Nabeshima *et al.*, 2022). The main path of *Bartonella* spp. transmission between vertebrate hosts is by blood-sucking arthropod vectors (Brenner *et al.*, 1993). Transmission can occur via the vector bite or its infected feces, the scratch or bite by the infected vertebrate hosts, or by contact with the infected blood (Raoult & Roux, 1999; Breitschwerdt & Kordick, 2000; Breitschwerdt *et al.*, 2007; Bouhsira *et al.*, 2013; Siamer & Dehio, 2015). In humans, bartonellae can cause (mainly zoonotic) diseases, bartonellosis, which are considered emerging infectious diseases (Breitschwerdt *et al.*, 2010; Guy *et al.*, 2013; Okaro *et al.*, 2017). Although only some *Bartonella* spp. are traditionally associated with human diseases (Breitschwerdt *et al.*, 2010; Battisti *et al.*, 2015; Gutiérrez *et al.*, 2017), it has been hypothesized that any species can have zoonotic potential (Breitschwerdt & Kordick, 2000; Okaro *et al.*, 2017).

The highest diversity of *Bartonella* species has been described in rodents acting as reservoirs with a prevalence reaching up to 90% worldwide (Bai *et al.*, 2007; Knap *et al.*, 2007; Tołkacz *et al.*, 2018; Mardosaitė-Busaitienė *et al.*, 2019a; Szewczyk *et al.*, 2020). Fleas associated with mammalian hosts have an important role in the life cycle of *Bartonella* sp. and are described to act as competent vectors and also as reservoirs of several species (Birtles, 2005; Brinkerhoff *et al.*, 2010; Morick *et al.*, 2013a).

Due to their longevity, high infestation rate with ectoparasites, and common interaction with humans (Matuschka *et al.*, 1994; Lipatova *et al.*, 2015; Schirò *et al.*, 2021), edible dormice (*Glis glis*) have the potential to play an important role in *Bartonella* spp. life cycle. However, the role of edible dormice so far remains unknown, although at least one study (Bundilová, 2020; diploma thesis) detected the presence of bartonellae. The description of the zoonotic pathogens in their natural host and their ecology is of interest and is necessary for the control and surveillance of emerging infectious diseases.

## 2 Literature review

### 2.1 *Bartonella* sp.

Representatives of the genus *Bartonella* are rod-shaped, small bacteria, on average around 0.3 µm in width and 1 µm in length (Minnick & Anderson, 2015). These Gram-negative bacteria are anaerobic and facultatively intracellular, transmitted between their vertebrate hosts by various arthropods (Brenner *et al.*, 1993). Human bartonellosis is considered to be an emerging infectious disease (Breitschwerdt *et al.*, 2010; Guy *et al.*, 2013; Okaro *et al.*, 2017).

#### 2.1.1 Taxonomy

The genus *Bartonella* belongs to the class Alphaproteobacteria (Maguiña *et al.*, 2009). Initially, the genus *Bartonella* was included in the order *Rickettsiales*. According to the current taxonomy, the genera *Rochalimaea* and *Bartonella* constitute the family *Bartonellaceae* within the order *Rhizobiales* (Brenner *et al.*, 1993; Madigan *et al.*, 2014). In recent years, there has been an increase in recognized species and genotypes of the genus *Bartonella* (Breitschwerdt, 2017; Tołkacz *et al.*, 2018). Altogether, 37 species and 3 subspecies of the genus *Bartonella* are currently described, another 28 species have an incomplete description (*Candidatus*) (Parte *et al.*, 2020), and some others are identified as novel species (only information about the phylogenetic position is available) (Murray & Stackebrandt, 1995; Han *et al.*, 2021; Majerová *et al.*, 2021).

#### 2.1.2 Vertebrate hosts and vectors

A wide variety of mammalian hosts have been described for *Bartonella* species, such as carnivores, rodents, bats, ungulates, and humans (Breitschwerdt, 2017; Iannino *et al.*, 2018; Neves *et al.*, 2018; Sacristán *et al.*, 2021; Nabeshima *et al.*, 2022). Moreover, *Bartonella* spp. have also been detected in marine vertebrates, such as sea otters, beluga whales, or sea turtles (Valentine *et al.*, 2007; Maggi *et al.*, 2008; Carrasco *et al.*, 2014). The large number and diversity of bartonellae hosts indicate a worldwide distribution (Vayssier-Taussat *et al.*, 2009; Gutiérrez *et al.*, 2015).

The main way of *Bartonella* sp. transmission between vertebrates is by arthropod vectors. To date, confirmed vectors are fleas (Chomel *et al.*, 1996), lice (Li *et al.*, 2013), ticks (Cotté *et al.*, 2008), sandflies of the genus *Lutzomyia* (Billeter *et al.*, 2008), keds (De Bruin *et al.*, 2015), Heteroptera of the genus *Cimex* (Leulmi *et al.*, 2015) and the role of mosquitoes as vectors is currently being discussed (Rudolf *et al.*, 2020).

The transmission of *Bartonella* sp. to vertebrates can occur via the vector bite and their infected feces. This way of transmission has been described, for example, in lice or fleas (Raoult & Roux, 1999; Bouhsira et al., 2013). Other possible ways of transmission are by the bite or scratch of an infected vertebrate host or by contact with infected blood (Breitschwerdt & Kordick, 2000; Breitschwerdt et al., 2007; Siemer & Dehio, 2015).

### 2.1.3 Clinical symptoms in humans

Bartonellae cause zoonotic, emerging infectious diseases in humans referred to as bartonellosis. The incidence of diagnosed bartonellosis increased in recent years, which is mainly due to more sensitive diagnostics (Okaro et al., 2017). Most cases are caused by three species: anthroponotic *B. bacilliformis* and *B. quintana*, and zoonotic *B. henselae* (Breitschwerdt et al., 2010; Battisti et al., 2015; Gutiérrez et al., 2017). The clinical manifestation can vary from mild symptoms such as headache, skin lesions, or fever to serious symptoms such as arthritis, meningitis, or encephalitis (Boulouis et al., 2005; Chomel et al., 2006). However, most infections of natural hosts are asymptomatic (Harms & Dehio, 2012).

*B. bacilliformis* is the causative agent of Carrion's disease which can be manifested in two different forms: verruga peruana (non-fatal cutaneous eruptions) or Oroya fever (generally fatal anemia) (Ihler, 1996). *B. quintana*, the causative agent of Trench fever, first described during World War I that was more recently observed in patients with human immunodeficiency virus (HIV), homeless people, or people abusing alcohol (Koehler et al., 1992; Brouqui et al., 1996). The described clinical symptoms of Trench fever are headache, leg pain, and thrombocytopenia (Brouqui et al., 1999). *B. henselae* causes cat-scratch-disease that is transmitted via scratch or less often the bite of a cat or some other infected mammals or vectors. Clinical symptoms are the formation of papules, lymphadenopathy, and fever (Carithers, 1985).

Given the high number of animal host reservoirs and vector species that can transmit bartonellae, it is discussed that the risk to humans and animals is likely to be underestimated (Breitschwerdt & Kordick, 2000). Furthermore, a high seroprevalence from 30 to 51% was shown in humans with a risk behavior in studies from Europe (Brouqui et al., 1999; Chmielewski et al., 2007). The combination of these factors with the proven high bacteremia in domestic and wild animals shows the great potential of bartonellae to cause emerging zoonotic diseases.

Although infections of the above-described species are well understood, a high zoonotic potential of other *Bartonella* species is discussed (Okaro et al., 2017). Zoonotic bartonellosis caused for example by rodent-associated *B. grahamii* was described to cause

symptomatic infections similar to the cat-scratch disease in immunosuppressed patients (Oksi *et al.*, 2013). *B. washoensis*, also detected mostly in rodents, was described to cause clinical symptoms (fever, myocarditis) in humans even in Europe (Kosoy *et al.*, 2003; Probert *et al.*, 2009; von Loewenich *et al.*, 2019).

#### 2.1.4 *Bartonella* sp. life cycle

Bartonellae are facultative intraerythrocytic parasites, this strategy helps them avoid the host's immune system. In addition, the blood localization enables transmission to blood-sucking arthropods. The development of *Bartonella* sp. in a vertebrate host begins when it is inoculated with a blood-sucking vector or transmitted by its infected feces (Fig. 1, Step A). Bacteria are not immediately able to colonize the erythrocyte, and to be able to do this step, they first need to infect the dermis and endothelial cells (Okujava *et al.*, 2014; Siamer & Dehio, 2015).

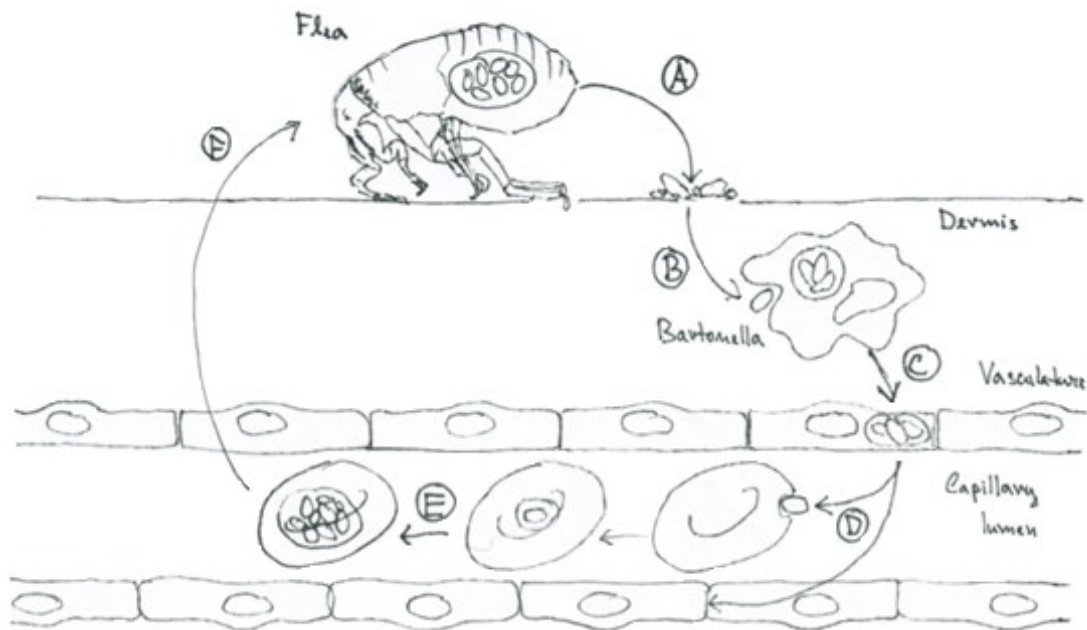


Figure 1: The development of *Bartonella* sp. in the host after the inoculation by infected vector. The bacteria first colonize the dermis and endothelial cells before being released into the bloodstream, infecting the erythrocytes. Based on Harms & Dehio, 2012.

First, bacteria are inoculated into the skin and colonize the dermal niche (Fig. 1, Step B) from where they are released to colonize the primary niche in endothelial cells, also referred to as the blood seeding niche (Fig. 1, Step C) (Okujava *et al.*, 2014; Siamer & Dehio, 2015). It is also discussed that *Bartonella* sp. can pass from the primary niche to other cells of the

reticuloendothelial system, such as migrating cells, e. g. dendritic cells. These cells could, upon their infection, help in the transmission of the bacteria to the sight of a new infection (Chomel *et al.*, 2009).

Bacteria are released into the bloodstream (Fig. 1, Step D) and they adhere to the erythrocytes, before invading them. On average, it takes four days for the bacteria to reach the bloodstream after the initial inoculation. After that, every three to six days a new wave of bacteria is released from endothelial cells and the bacteria synchronously colonize erythrocytes (Koesling *et al.*, 2001; Schülein *et al.*, 2001; Okujava *et al.*, 2014).

Bartonellae replicate in the erythrocytes in the membrane vacuole (Fig. 1, Step E) and when reaching a certain density, the replication stops (Schülein *et al.*, 2001). The bacteremia is in this way maintained for approximately ten weeks (Koesling *et al.*, 2001; Okujava *et al.*, 2014). If reinfection from the endothelial cell is prevented, intraerythrocytic bacteremia lasts for the lifespan of infected erythrocytes (Koesling *et al.*, 2001). In addition to reinfections, *Bartonella* sp. can also persist in endothelial cells for a long time, which is referred to as the dormant stage. The bacteria are not released into the bloodstream. This stage can last from weeks to years until reactivation and a new release of bacteria into the bloodstream (Pulliainen & Dehio, 2012).

#### **2.1.5 *Bartonella* spp. mixed infections**

Interaction between bacteria of various species can lead to horizontal gene transfer (HGT), which is however more common between bacteria of the same species (Smith *et al.*, 1993; Wiedenbeck & Cohan, 2011). HGT can significantly affect the evolutionary potential and diversity of bacteria and can lead to the emergence of new species and the blurring of species boundaries by bacterial recombination (Welch *et al.*, 2002; Wiedenbeck & Cohan, 2011). Bartonellae have more opportunities in ways to interact with other bacteria because they are transmitted by vectors and therefore colonize two different environments during their development (Cirimotich *et al.*, 2011; Hawlena *et al.*, 2013; Gutiérrez *et al.*, 2018). Mixed infections in vertebrate hosts and invertebrate vectors with multiple *Bartonella* species were repeatedly reported (Abbot *et al.*, 2007; Brinkerhoff *et al.*, 2010; Chan & Kosoy, 2010; Gutiérrez *et al.*, 2018; Tołkacz *et al.*, 2018) and it was proposed that mixed infections are common rather than a rare phenomenon. The rate of mixed infections of different genotypes suggests that ongoing homologous recombination between genotypes and species promotes the evolution of bartonellae (Gutiérrez *et al.*, 2018).

The infection of the host with more species of *Bartonella* at the same time was described to have a relapsing character. One species can be dormant in the primary niche and not be detectable through blood analysis while the other one is active and replicating in erythrocytes. The active species can become dormant and re-activation of the other one, the so-far dormant species, can occur (Kabeya *et al.*, 2002).

#### **2.1.6 Methods of *Bartonella* sp. detection and determination**

Bartonellae infections can be detected by a variety of methods ranging from molecular methods to serology. The direct methods of *Bartonella* sp. detection are cultivation and polymerase chain reaction (PCR) which are described in detail further, and indirect methods using host sera such as fluorescent antibody assay (IFA) or enzyme-linked-immunosorbent-assay (ELISA), which were not used in this study and therefore will not be further discussed (Agan & Dolan, 2002; Okaro *et al.*, 2017).

##### **2.1.6.1 Cultivation of bacteria**

Because bartonellae are facultatively intracellular and during their development in the host invade erythrocytes, the bacteria are usually cultured on medium-enriched by blood or hem supplements (Minnick & Anderson, 2015). The best growth was observed in the media containing from 5 to 10% of rabbit, horse, or sheep blood in an environment with 5% CO<sub>2</sub>, and even so, the bacteria are considered fastidious and slow-growing (Myers *et al.*, 1969; Brenner *et al.*, 1993; Minnick & Anderson, 2015). The optimal cultivation temperature ranges from 35 to 37 °C, or 25 to 28 °C for *B. bacilliformis* (Brenner *et al.*, 1991, 1993; Fournier *et al.*, 2007; Mullins *et al.*, 2015). Apart from the different optimal cultivation temperatures, *B. bacilliformis* also does not require the presence of added CO<sub>2</sub> (Brenner *et al.*, 1991). Research showed the possibility to culture some *Bartonella* species on liquid media derived from Schneider's insect media (Riess *et al.*, 2008).

Better growth was described on rabbit or sheep blood media compared to the chocolate agar (containing hemoglobin solution) for *B. quintana* (Welch *et al.*, 1992). Some studies discuss the possibility of replacing blood with hemin, starch, or charcoal (Myers *et al.*, 1969, 1972; Welch *et al.*, 1992). The bacteria of the genus *Bartonella* require a surprisingly high amount of hem in media for their growth, which suggests its function as a precursor for the synthesis of proteins with hem (Myers *et al.*, 1972).

#### **2.1.6.2 Determination of *Bartonella* species**

Identification of *Bartonella* species is considered to be challenging because of the fastidious nature of the bacteria and, therefore, difficult cultivation *in vitro* (Gutiérrez *et al.*, 2017; Vaca *et al.*, 2022). Although molecular techniques became quite proficient in *Bartonella* sp. detection over time, they are still not accurate enough due to the high amounts of mixed infections of different *Bartonella* species (Regnery *et al.*, 1992; Abbot *et al.*, 2007; Gutiérrez *et al.*, 2017). To detect mixed infections, detailed analysis is needed by both molecular and cultivation methods (Gutiérrez *et al.*, 2017)

##### **2.1.6.2.1 Molecular methods**

Conventional PCR (one-step) is used for the detection of bartonellae infections from clinical samples; however, this method is not so suitable for the identification of subclinical infections of wild animals and/or vectors (Birtles *et al.*, 2000; Rampersad *et al.*, 2005). In free-living animals, the studies are carried out as screenings, a lower number of bacteria is present and therefore more sensitive methods such as real-time or nested PCR are recommended for detection. However, these methods have lower specificity due to the smaller size of amplicons. The specificity issue can be avoided by combinations of multiple loci analysis or complementary analysis with conventional PCR (Rampersad *et al.*, 2005; Diaz *et al.*, 2012; Gutiérrez *et al.*, 2017). The targets for *Bartonella* spp. identification are mostly housekeeping loci such as the citrate synthase gene (*gltA*) and the gene coding the beta-subunit of RNA polymerase gene (*rpoB*) that are advantageous in their stability and because they are the point of interest of other studies (Gutiérrez *et al.*, 2017). However, PCR is prone to detect the most dominant species in the samples, and therefore it is advisable to use PCR of several loci (Gutiérrez *et al.*, 2013, 2014a). Different primers can be more specific to other species and therefore mixed infections are more likely to be discovered (Whiley *et al.*, 2008).

Because the detection and identification of *Bartonella* species require detailed analysis, several recommendations were published for the procedure: real-time PCR should be preferred over conventional PCR methods, obtained PCR products should be sequenced, positive samples should be targeted for additional analysis of different loci, one-step PCR should be used to obtain long products and before analysis of samples from new hosts or vectors, the specificity of primers should be determined (Gutiérrez *et al.*, 2017).

#### 2.1.6.2.2 MALDI-TOF

A more recently described method of bartonellae identification is Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) which is otherwise used for routine bacteria identification of clinical samples (Bizzini & Greub, 2010).

The reliability of MALDI-TOF identification was tested on 1660 bacterial isolates and the isolates were correctly identified in 84.6% at the species level and 95.4% at the genus level (Seng *et al.*, 2010). The major advantage of this identification technique is the cost-effectiveness ratio, not counting only the cost of the procedure but also the preparation of samples and growth of cultures (Bizzini & Greub, 2010; Seng *et al.*, 2010). MALDI-TOF is easy to use and compared to molecular techniques is not as time-consuming (Bizzini & Greub, 2010).

However, the database library of MALDI-TOF so far contains only a small number of organisms compared to the diversity found in nature. This problem is less pronounced in human pathogens because clinical microbiology focuses on these samples (Liébana-Martos, 2018). It is recommended, that the bacterial colonies used for identification should be as fresh as possible (growth age up to 48 hours), because of ribosomal protein degradation, less distinguished peaks would be otherwise measured (Chean *et al.*, 2014; Liébana-Martos, 2018). However, even slow-growing bacteria were successfully identified via MALDI-TOF analysis (Biswas & Rolain, 2013). Furthermore, the absence or presence of certain peaks and their intensity can be subject to analytical error and variance in protein expression, modifications, and regulations. Although a high species specificity was observed, generalization should be made with care, as mass spectral patterns of certain species show increased variability (Giebel *et al.*, 2010; Liébana-Martos, 2018).

Traditionally, MALDI-TOF identification was performed only on cultivated microbiological samples (Bizzini & Greub, 2010). Direct identification of samples without cultivation was successfully performed for example on blood or urine clinical samples (La Scola & Raoult, 2009; Bizzini & Greub, 2010; Ferreira *et al.*, 2010). This method was used to successfully identify cultivated bacteria of the genus *Bartonella*, identify 39 strains at the species level, and recognize several subspecies (*B. vinsonii vinsonii*, *berkhoffii*, and *arupensis*) (Fournier *et al.*, 2009). Some studies tested a protocol of *Bartonella* sp. detection via MALDI-TOF without prior cultivation from homogenized fleas following a described protocol (Nebbak *et al.*, 2017; El Hamzaoui *et al.*, 2018).

### 2.1.6.3 Procedure for a description of new species

To describe a new isolate as a new bacterial species, several requirements must be met. For a valid species description, a detailed characterization describing the distinguishing traits of the species must be published (Heller *et al.*, 1999; Madigan *et al.*, 2014; Trujillo *et al.*, 2017).

Studies on bartonellae describe the possibility to perform phylogenetic analysis from the sequences of different loci such as *gltA*, *rpoB*, cell division protein (*ftsZ*), internal transcribed spacer (ITS), riboflavin synthetase (*ribC*), and GroEL chaperonin (*groEL*) (Birtles & Raoult, 1996; Houpikian & Raoult, 2001; Zeaiter *et al.*, 2002; La Scola & Raoult, 2009). It was proposed that a new (= distinct) species should be recognized by the sequence identity of the partial *gltA* gene (327 bp) lower than 96% alongside the partial *rpoB* locus (825 bp) identity lower than 95% (La Scola *et al.*, 2003).

Furthermore, the sequence of 16S rRNA should be studied and the similarity to other species observed. The described species should be characterized by its morphological and physiological characteristics. The morphology of cultivated colonies as well as the morphology of bacterial cells should be described. Biochemical analysis should be performed to characterize the species. In addition, whole-genome sequencing of the newly described species is recommended. The type strain of viable culture should be placed in at least two international culture collections (Heller *et al.*, 1999; Madigan *et al.*, 2014; Trujillo *et al.*, 2017).

### 2.1.7 Rodent reservoirs of bartonellae

Rodents as reservoir hosts for different *Bartonella* species have been extensively studied (Gutiérrez *et al.*, 2015). The studies of multiple rodent species estimated the prevalence of bartonellae in different parts of the world to reach up to 90% (Bai *et al.*, 2007; Knap *et al.*, 2007; Totkacz *et al.*, 2018; Mardosaitė-Busaitienė *et al.*, 2019a; Szewczyk *et al.*, 2020). However, the prevalence of *Bartonella* spp. varies a lot between different rodent species, as was described for example in Denmark (Engbaek & Lawson, 2004). On the other hand, some rodent species showed to be less susceptible to bartonellae or even completely resistant (Bai *et al.*, 2007).

Among the mammalian hosts, the greatest diversity of *Bartonella* species was described from rodents. In Europe, the following species have been detected: *B. birtlesii*, *B. coopersplainsensis*, *B. doshiae*, *B. elizabethae*, *B. grahamii*, *B. henselae*, *B. phoceensis*, *B. rattimassiliensis*, *B. rochalimae*, *B. taylorii*, *B. tribocorum*, *B. vinsonii* subs. *vinsonii*, and *B. washoensis* (Bermond *et al.*, 2000; Engbaek & Lawson, 2004; Gundi *et al.*, 2004; Knap *et al.*, 2007; Špitalská *et al.*, 2017; Mardosaitė-Busaitienė *et al.*, 2019a; Majerová *et al.*, 2021).

It is discussed that the diversity of bartonellae in rodents is caused by adaptive radiation as most of the species discovered in rodents are clustered into one lineage with few exceptions (*B. rochalimae*, *B. tamiae*) (Engel *et al.*, 2011; Buffet *et al.*, 2013). The number of recognized *Bartonella* species from rodents has rapidly increased over the recent years, which could be caused by horizontal gene transfer, mutation accumulation, frequent homologues recombination, or the focus of *Bartonella* sp. research on rodents (Berglund *et al.*, 2010a; Guy *et al.*, 2013; Gutiérrez *et al.*, 2015). The frequency of recombination and HGT is typically low for intracellular bacteria as they are isolated (Vos & Didelot, 2009). The recombination and HGT frequency differ also between different lineages of bartonellae with higher frequency for *Bartonella* spp. in rodents. This difference, and overall higher-than-expected frequency of recombination and HGT events, could be due to the mixed infections, high rodent abundance, and higher vector exposure (Berglund *et al.*, 2010a).

## **2.2 Dormice**

Dormice are small rodents of the family Gliridae that belong according to a current classification into the group Sciuromorpha together with squirrels and mountain beavers (Barthelmess *et al.*, 2016). Four species of dormice occur in the Czech Republic: garden dormouse (*Eliomys quercinus*), common dormouse (*Muscardinus avellanarius*), forest dormouse (*Dryomys nitedula*), and edible dormouse (*Glis glis*) (Andera, 1994).

### **2.2.1 Biology of edible dormice**

Edible dormice are small rodents with an average total body and a tail length of 390 mm, where the length of the tail is usually about 80% of the body length (Barthelmess *et al.*, 2016). Edible dormice are mostly distributed in Asia Minor and Europe (from northern Spain to the River Volga, the most northern locations are Baltic states and southern Cyprus; Fig. 2). In the south, they are considered to be pests, whereas in the north their populations are threatened (Mitchell-Jones *et al.*, 1999).



Figure 2: The distribution of edible dormice (*Glis glis*) in Europe and Asia according to Amori et al., 2021.

Edible dormice are inhabitants of deciduous and mixed forests, which is the reason for their mosaic distribution in the Czech Republic corresponding to the fragmentary occurrence of their preferred habitat in the country (Andera, 1994). Their populations are adapted to fluctuations in the food resources during the year, fluctuations in seed production depending on the year, and fluctuations in temperature and precipitation during the day (Ruf & Bieber, 2020). Although most animals are affected by similar fluctuations in time, the effects are more pronounced on animals as small as edible dormice. Furthermore, oak and beech have mast seeding, and the availability of their seeds changes between years. Because oak and beech seeds are the main food source for dormice, their populations are affected by fluctuating seed availability (Barthelmess *et al.*, 2016; Ruf & Bieber, 2020). Other food sources include leaves and fruits (Fietz *et al.*, 2005), and the role of dormice as predators of nesting birds is discussed (Adamík & Král, 2008a). Edible dormice are highly dependent on the high energetic seed availability for reproduction, which is positively correlated with the number of seeds from the oak and beech (Bieber, 1998).

To survive the weather conditions fluctuations, the edible dormice are obligatory hibernators during winter, active only for about four to six months per year (Fietz *et al.*, 2004; Ruf & Bieber, 2020). Dormice hibernate below ground, even as deep as 60 cm (Hoelzl *et al.*, 2015), and because of that their preferred substrate is karstic with underground spaces for hibernation (Mitchell-Jones *et al.*, 1999).

Even during the rest of the year, when they are active, summer torpor was observed, but only in non-reproductive years (Fietz *et al.*, 2004; Ruf & Bieber, 2020). Edible dormice have

nocturnal activity and during the day they can be mostly found sleeping in nest boxes and the hollow trees (Adamík & Král, 2008a; Madikiza *et al.*, 2010). They usually occupy the nest boxes from the end of April or May until the middle of October (Trilar, 1997).

On the other hand, if the conditions are unfavorable and the dormice have enough body mass, they can hibernate again only a few weeks after waking up from the last hibernation. As they can again hibernate until spring, they can sleep even 19 out of 24 months (Bieber & Ruf, 2009; Hoelzl *et al.*, 2015; Ruf & Bieber, 2020). The ability for long-term hibernation contributes to the unusually high longevity of dormice which is reported to be up to nine years. Most females reproduce once or twice per lifetime and mostly in mast seeding years when there are enough resources for reproduction (Schlund *et al.*, 2002; Pilastro *et al.*, 2003).

The edible dormice were in the past used as a food source (and in some regions, they are used even today), which corresponds to their name. Although dormice are no longer kept at home, they are still hunted for their meat (Carpaneto & Cristaldi, 1994; Krystufek *et al.*, 2003). When the abundance of dormice rises, they are considered pests in both agriculture and forestry due to damage to tree barks and fruits and crops consummation. Furthermore, they cause a decrease in birds' population because of predation of nestlings and taking over places of birds' nesting sites. Dormice tend to enter human homes and inhabit their roofs or live near rural houses and cottages, which they seek out for shelter and access to food (Carpaneto & Cristaldi, 1994; Adamík & Král, 2008a; Trout *et al.*, 2015).

### **2.2.2 Dormice as hosts of various pathogens**

The potential of dormice to transmit pathogens to domestic animals and humans is not yet well understood (Lipatova *et al.*, 2015). Due to their high infestation levels with both fleas and ticks, it is discussed that the edible dormice could potentially be important reservoir hosts for zoonotic diseases carried by these vectors (Lipatova *et al.*, 2015; Schirò *et al.*, 2021). They seem to be one of the most competent reservoir hosts of *Borrelia burgdorferi*, due to their unusual longevity and high infestation rate with ticks (Matuschka *et al.*, 1994). Furthermore, the pathogens *Rickettsia typhi*, *Anaplasma phagocytophilum*, *Borrelia miyamotoi*, and *Borrelia afzelii* were detected in samples from edible dormice (Trilar *et al.*, 1994; Kraljik *et al.*, 2017). In addition, some species of helminths that were described to be able to infect humans were detected in edible dormice, for example, *Mesocostoides lineatus* or *Hymenolepis diminuta* (Eom *et al.*, 1992; Konjević *et al.*, 2007; Schirò *et al.*, 2021). The role of dormice as hosts of emerging viruses, such as hantavirus and monkeypox virus, is discussed (Earl *et al.*, 2015; Stanojevic *et al.*, 2015).

## 2.3 Fleas

Fleas (order Aphaniptera or Siphonaptera) are worldwide blood-sucking ectoparasites of mammals and birds with the majority of species (95%) found on mammals (Brusca & Brusca, 2003; Resh & Cardé, 2003; Mehlhorn, 2012). Fleas have four different developmental stages: egg, larva, pupa, and adult (Fig. 3). The number of larval instars and laid eggs, their location as well as the length of their life cycle differs according to flea species. The timing of changes between the various stages of flea development is usually synchronized with the reproduction and life cycle of the host (Resh & Cardé, 2003).

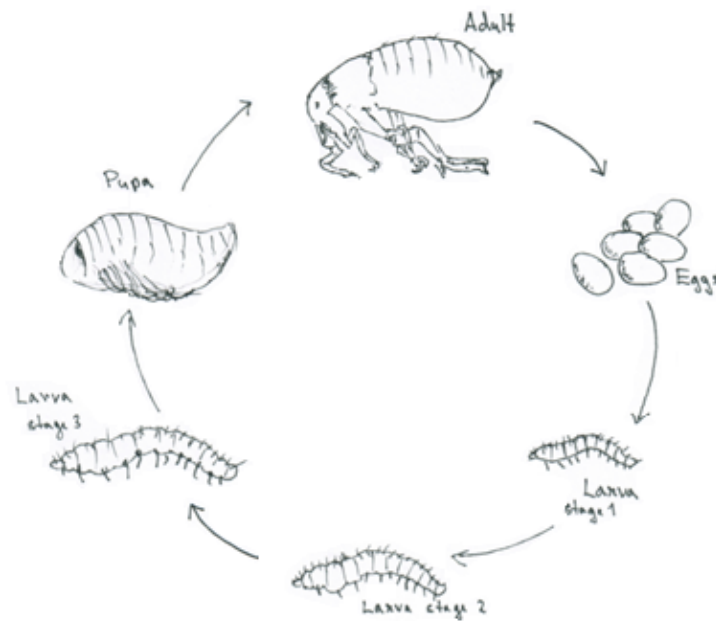


Figure 3: The lifecycle of fleas (holometabolous insect) with different developmental stages eggs, larva, pupa, and adult. Based on Dobler & Pfeffer, 2011.

There are typically three larvae instars that are about 1.5 to 10 mm long without a head, legs, or eyes (Resh & Cardé, 2003). The larvae's food source is organic debris that is located in the vicinity of the host's nest (Mehlhorn, 2012). The adult fleas do not feed just to satisfy their requirements, but they also excrete superfluous blood in their feces, which is afterward eaten by larvae (Brusca & Brusca, 2003; Mehlhorn, 2012).

Fleas are not particularly host-specific, and their specificity is more dependent on the requirements of the larvae stages than on adult fleas. Apart from that, adult fleas change their hosts freely and it is described that they can change hosts daily (Brusca & Brusca, 2003; Mehlhorn, 2012).

### 2.3.1 Fleas as vectors

To be a successful vector for zoonotic diseases, fleas must satisfy several requirements: an adequate amount of the pathogen in the biotope populated by fleas, enough possibilities of transmission of fleas between animals and humans, survival, and reproduction (if possible) of the pathogen in fleas' mouthparts or gastrointestinal tract, and a large number of fleas that switch hosts often.

The transmission of pathogens can occur via the contaminated mouthparts of fleas and by regurgitation of blood (Mehlhorn, 2012). Fleas are the vectors for zoonotic bacteria such as *Yersinia pestis* (causative agent of plague), *Rickettsia typhi* (murine typhus), *Francisella tularensis* (tularemia), *Listeria monocytogenes* (listeriosis), *Salmonella* spp. (salmonellosis), *Coxiella burnetti* (Q fever), and *Bartonella henselae* (cat scratch disease) (Resh & Cardé, 2003; Mehlhorn, 2012). In addition, fleas are intermediate hosts for several helminths such as tapeworms (*Dipylidium*, *Hymenolepis* species) and some nematodes (*Dipetalonema* group) (Mehlhorn, 2012).

A papule is formed on the site of a flea bite that is caused by a response to the injection of saliva, furthermore intense itching for several days is described. More severe clinical symptoms may occur in the case of bacterial superinfections, such as sepsis and severe inflammation. Some species of fleas (*Tunga penetrans*) can cause disease directly when entering their hosts (Mehlhorn, 2012).

#### 2.3.1.1 Fleas and *Bartonella* spp.

Fleas are considered to have an important role in the life cycle of *Bartonella* spp. as they are competent vectors for a wide spectrum of *Bartonella* species and genotypes (Brinkerhoff *et al.*, 2010; Morick *et al.*, 2013b). Bartonellae infection of fleas appears to be not pathogenic for fleas and does not influence their metabolic rate, longevity, fertility, fecundity, and size of blood meal; therefore, it is discussed that fleas are well adapted to *Bartonella* sp. presence because of long-term coevolution (Bouhsira *et al.*, 2013; Morick *et al.*, 2013c) and could be considered not only vectors but also (additional) reservoir hosts for some *Bartonella* species (Birtles, 2005).

The bacteria replicate in the gut of a flea and are secreted out with the feces (Finkelstein *et al.*, 2002). During the lifetime of fleas, a decrease in the number of secreted bacteria in the feces was observed, therefore it is discussed that maybe the bacteria bartonellae change their localization from the gastrointestinal tract to a body cavity or other organs such as hemolymph or reproductive organs (Higgins *et al.*, 1996; Brinkerhoff *et al.*, 2010; Bouhsira *et al.*, 2013). Additional research is needed to establish the possibilities of bacteria reproduction out of the gastrointestinal tract of fleas (Bouhsira *et al.*, 2013). The

possibility of bacteria staying viable in the feces in the outer environment for a minimum of three days (thanks to the biofilm formed on the surface of feces) is currently discussed (Finkelstein *et al.*, 2002; Seki *et al.*, 2007; Bouhsira *et al.*, 2013). The differences in the transmission efficiency for diverse *Bartonella* species with diverse flea species are so far unknown. However, these differences could be important to know, as well as the ability of fleas to transmit different *Bartonella* species within mixed infections (Bown *et al.*, 2004).

The detection of bartonellae from the reproductive tissue of fleas suggests possible vertical transmission that in combination with host switching could lead to maintenance of different, even rare, *Bartonella* species (Brinkerhoff *et al.*, 2010). The vertical transmission in fleas was so far confirmed only as non-transovarial transmission through the infected flea feces on which the larvae feed (Morick *et al.*, 2013a). Furthermore, the transovarial transmission was tested for the flea *Xenopsylla ramesis* and the results showed that the transmission does not occur (Morick *et al.*, 2011).

### 2.3.2 Fleas of dormice

Dormice can get infected with fleas from other ecological groups while roaming through the locality and while nesting in a nest previously occupied by birds. It was described that dormice can come to contact with several different communities of fleas: fleas of small birds, red squirrels, rodents, and other small mammals (Rosický, 1957; Lipatova *et al.*, 2015). On edible dormouse and their nests, altogether 14 species of fleas were detected in Central Europe (Trilar, 1997; Brelih & Trilar, 2001).

The dominant flea of edible dormice is *Ceratophyllus (Monopsyllus) sciurorum* (Trilar, 1997; Lipatova *et al.*, 2015; Bundilová, 2020), which is a red squirrel flea found on various small mammals, nest birds, and even humans (Rosický, 1957). The flea of the subgenus *Monopsyllus* was detected on several species from the order *Gliridae* (Brelih & Trilar, 2001; Büchner *et al.*, 2003). *Ceratophyllus gallinae* is a species of flea that often infect birds nesting in nest boxes or hollow trees (Trilar, 1997; Büchner *et al.*, 2003) and is also described in dormice, squirrels, and humans (Rosický, 1957). An extensive study was carried out on edible dormice nests in Slovenia describing all ectoparasites in altogether 57 nests. Although other species of ectoparasites such as the lice *Schizophthirius* sp. and *Cimex (Oeciacus) hirundinis* were described, the most prevalent group of ectoparasites were fleas and the most prevalent species was *C. sciurorum*, forming 96% of all ectoparasites (Trilar, 1997).

Although a high number of fleas was detected in nests, no adverse effects on the brood size of edible dormice were observed (Trilar, 1997). However, negative effects of unnatural high ectoparasites load on rodents were described to cause irritation and damage to the host skin, injections of saliva toxins as well as pathogens, and loss of body mass due to energy cost of immune and behavior response (Hawlana *et al.*, 2006).

### 3 Aims of the study

1. To determine the prevalence and diversity of *Bartonella* spp. in the blood of dormice and their fleas
2. To study the bartonellae infection in different life stages of fleas
3. To obtain and characterize cultures of *Bartonella* spp.

To determine the prevalence and diversity of *Bartonella* spp., samples obtained from the seasons 2016 to 2021 were analyzed. Dormice blood samples and fleas collected directly from animals, or their nests were used for specific PCR detection of the loci *gltA*, *rpoB*, *ftsZ*, and ITS, and positive samples were sequenced. The reliability of identification by different loci was compared and their suitability was determined.

Fleas at various stages of their development (primary adult fleas in nests, larvae, and newly emerged adult fleas) were analyzed for the presence of *Bartonella* DNA by PCR (ITS). Newly emerged fleas and flea feces were cultivated to test the viability of bacteria.

The samples of dormice blood and homogenized fleas were cultured and detected *Bartonella* species (by PCR and/or MALDI-TOF) were passaged to obtain clones of *Bartonella* strains. Different species/genotypes were characterized, including colony and cell morphology (transmission electron microscopy), and the preference in temperature and presence of CO<sub>2</sub> for growth.

## 4 Materials and methods

The edible dormice (*Glis glis*) and their fleas were captured in a locality in North Moravia, in the district of Olomouc close to the municipality Dlouhá Loučka (Fig. 4). At this locality, long-term ecological projects on the edible dormice population are taking place. The sampling was conducted in collaboration with Peter Adamík, Ph.D. (Palacký University Olomouc) authorized to handle and work with the protected species, and the veterinarian, prof. David Modrý (University of Veterinary and Pharmaceutical Sciences, Brno).

The material from the years 2016 to 2018 was obtained for the MSc project of Kateřina Bundilová (Bundilová, 2020), where preliminary data about *Bartonella* spp. occurrence were presented. The material contained extracted DNA of dormice blood (2016–2018) and fleas from dormice (2017–2018).

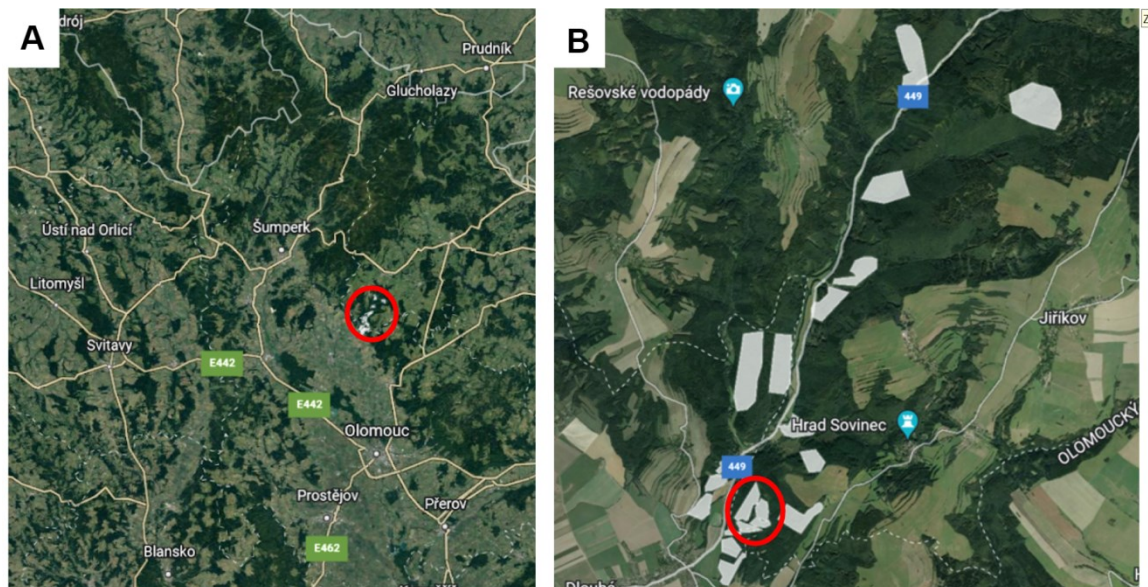


Figure 4: A: The location of the sampling locality (red circle) near Dlouhá Loučka in the Olomouc district. B: The location of 20 sampling sites; three sites with the majority of samplings are highlighted in red. Source: taken from (earth.google.com).

### 4.1 Field sampling and laboratory processing

Long-term observations of edible dormice in the locality are carried out annually from June to October when the dormice are awoken from hibernation and using nest boxes. In 2016, blood was taken from dormice in July and September and no fleas were sampled. In 2017 and 2018, the samplings of fleas from dormice were conducted every month from June to October without dormice narcotizing, blood was taken from dormice in July and August in 2017 and July and September in 2018 (Bundilová, 2020). In 2019, the sampling was conducted in August and September of both blood of dormice and fleas. In 2020, field sampling took place in

August, when blood was collected from narcotized dormice, fleas, and nests were collected from nest boxes. In 2021, the sampling in July was conducted without the assistance of a veterinarian, who would narcotize dormice; therefore, no blood samples were taken, and fleas were removed in smaller numbers. In addition, nests were collected from each nest box occupied by the dormice. In August 2021, additional samples were taken, the blood was sampled, fleas were removed from dormice, and nests were collected<sup>1</sup>.

There are a total of 20 sites in the study locality, sampling in the years 2016 to 2018 was performed mainly on seven of them (Bundilová, 2020). However, sampling in 2019 took place at six sites, and in 2020 and 2021 sampling took place mainly at 3 sites, although other localities were also checked for the presence of dormice (July 2021).

Edible dormice were removed from the nest boxes, which were located at a height of 1.5 to 2 meters on trees. Most of the nest boxes in the region were designated for small birds such as birds of the genus *Parus* s. l. However, a small number of nest boxes were intended for hazel dormice (*Muscardinus avellanarius*) with the entrance of the nest box oriented towards the tree.

#### **4.1.1 Dormice and flea sampling**

The sampling of dormice in the years 2020–2021 was conducted during the day when dormice were asleep in the nest boxes. Capturing and manipulating with awakened dormice was performed by Peter Adamík. The sex of the dormice as well as its age and measurements were determined, and the dormice were checked for the presence of an identification veterinary microchip. If the microchip was not present, a new microchip was given to the dormice and the dormice were put into a cloth bag. Afterward, one by one they were taken out and narcotized (by David Modrý) by intramuscular application of narcotics. After a few moments, the blood was sampled from inferior *vena cava cranialis* using a sterilized hypodermic needle. Sampled blood was either combined with 100 µl of Ethylenediaminetetraacetic acid (EDTA) for cultivation or with 100 µl sodium dodecyl sulfate (SDS) for DNA isolation, in 1.5 ml Eppendorf tubes and transported to the laboratory on the dry ice.

While the dormice were still narcotized, each dormouse fur was brushed for ectoparasite removal. Fleas were removed using tweezers and put individually into 1.5 ml Eppendorf tubes filled either with 70% ethanol or into empty ones. Fleas collected into tubes with ethanol were designated for PCR analysis only, whereas fleas put into empty tubes were

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<sup>1</sup> I personally participated in the samplings in 2020 and 2021.

kept alive for bacteria cultivation. The fleas were transported to the laboratory for further analysis. From each nest box containing at least one dormouse, the nests were collected into plastic bags for further analysis of fleas.

Thereafter, the dormice were awoken by Revertor that reversed the effect of the narcotics. Awakened dormice were then put back into the nest boxes. During field sampling in July and August 2021, fleas removed from dormice were partly processed in the field. They were for a few seconds washed in 70% ethanol for surface cleaning and dried with cellulose paper towels. Decontaminated fleas were put into separate sterilized 1.5 ml Eppendorf tubes and transported to the laboratory for further analysis.

## 4.2 Processing of samples

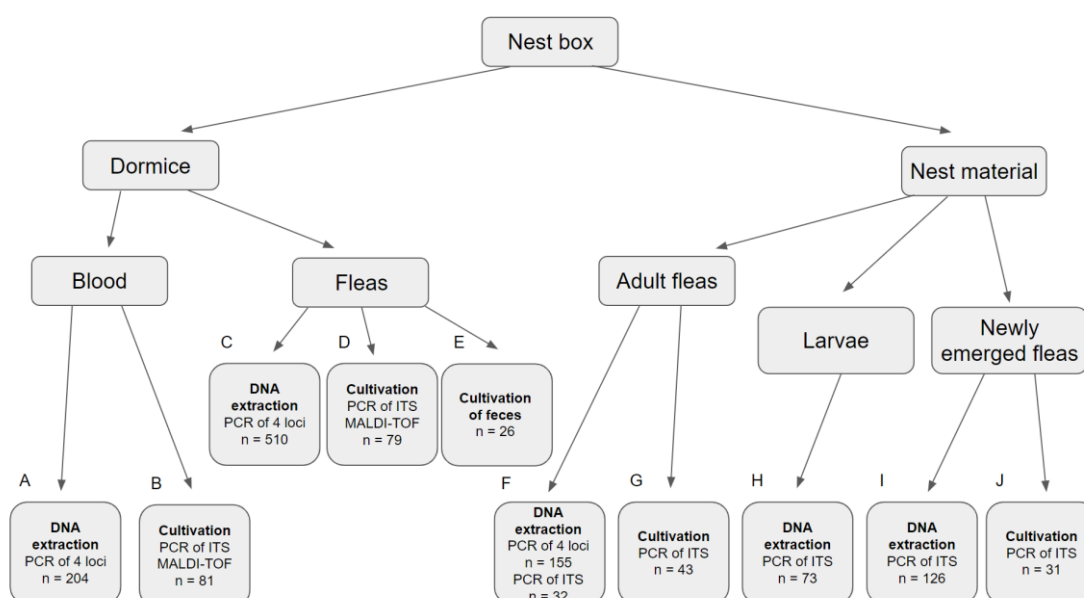


Figure 5: Scheme describing different steps of processing of samples taken from dormice and nest boxes, with the number of samples used for different parts of the analysis.

### 4.2.1 DNA extraction

The samples used for DNA extraction were dormice blood samples in SDS, the other half of the homogeneous flea solution from the preparation for cultivation, fleas removed from dormice and nests, newly emerged adult fleas, and larvae designated just for DNA extraction and PCR analysis. For DNA extraction, the DNeasy Blood & Tissue kit (QIAGEN) was used according to the provided manual. Only the last step was adjusted and instead of 200 µl of elution buffer, 100 µl of elution buffer diluted in the ratio of 1:1 with PCR grade water was used for a higher yield of DNA.

#### **4.2.2 Blood of dormice**

The samples of dormice blood in both EDTA for cultivation and SDS for DNA extraction (Fig. 5, A) were stored at  $-20^{\circ}\text{C}$  upon arrival at the laboratory. The samples designated for cultivation were stored for up to several months before cultivation. 100  $\mu\text{l}$  of cultivation medium Schneider's insect medium based (SIMB) was added to the blood samples directly before cultivation (Fig. 5, B).

The SIMB medium was prepared by adding fetal calf serum (FCS) (10%), saccharose (5%), and Amphotericin B (0.1%) to the Schneider's Insect Medium (Sigma Aldrich). The mixture with dissolved saccharose was filtered by a 0.22 filter (MILEX) into a new tube before use.

#### **4.2.3 Fleas collected directly from dormice**

The fleas from dormice that were meant for cultivation (Fig. 5, D) were stored at a temperature of  $4^{\circ}\text{C}$  for up to a week upon their arrival at the laboratory to keep them alive. Before cultivation, the fleas were killed by freezing for 5 minutes at  $-20^{\circ}\text{C}$ . Determination of flea species as well as sex of fleas was conducted using a binocular magnifier (ZEISS SteREO Discovery.V8) on a petri dish in physiological saline solution (1x PBS). The fleas were washed in 70% ethanol for surface sterilization and then three times in sterile PBS. Sterilized pestles were used for homogenization of fleas in 100  $\mu\text{l}$  of SIMB medium. Half of the resulting homogeneous mixture was used for cultivation and the other half for DNA extraction.

The fleas from dormice used only for DNA extraction (Fig. 5, C) were stored long-term at  $-20^{\circ}\text{C}$ . Up to ten fleas per dormouse were used, and each flea was analyzed separately. The species and sex of fleas were determined as described above. The fleas were directly homogenized by sterilized pestles in 180  $\mu\text{l}$  of lysis buffer ATL from an isolation kit (Qiagen).

#### **4.2.4 Fleas occupied the dormice nests**

The nests collected during the dormice sampling were stored for up to several months at  $4^{\circ}\text{C}$ . For analysis, the nests were searched for adult fleas that were removed with tweezers and placed in 1.5 ml Eppendorf tubes. The tubes with fleas were stored for up to a week at  $4^{\circ}\text{C}$  if used for bartonellae cultivation (Fig. 5, G) or long-term storage at  $-20^{\circ}\text{C}$  if used for DNA extraction only (Fig. 5, F). Fleas for cultivation and DNA extraction were prepared as described above; up to 10 fleas from each nest were used for analysis, and each flea was processed separately.

#### **4.2.5 Detection of bartonellae during the lifecycle of fleas**

##### **4.2.5.1 Fleas from the dormice nests**

The nests collected during the dormice sampling were stored at 4 °C for up to several months upon their arrival at the laboratory. In the year 2020, the experiment was carried out on winter nests. The absence of primary adult fleas in winter nests was expected and the nests were left at room temperature for several days. The nests were checked repeatedly every week for newly emerged adult fleas. This experiment was a pilot one and its main result was testing methods for handling fleas in nests. The experiment was then performed on nests collected during the summer of 2021.

The next year 2021, the nests from both July and August 2021 field samplings were used. The nests were stored at 4 °C for up to several months to ensure the suspension of the flea life cycle. The nests were taken out of the fridge and all the primary adult fleas (Fig. 5; F, G) with up to 10 larvae (Fig. 5, H) were taken out for further analysis. The samples were stored at -20 °C because were used only for DNA extraction. Before analysis, the species of primary adult fleas were processed as described above.

The nests were from then on stored at room temperature and every week they were searched for newly emerged adult fleas. The newly emerged fleas were collected into 1.5 ml Eppendorf tubes and stored for up to a week in the fridge. Part of the newly emerged fleas was used for cultivation (Fig. 5, J) as well as DNA extraction, others were used only for DNA extraction (Fig. 5, I) as described above.

##### **4.2.5.2 Adult flea feces**

Decontaminated fleas, which were pre-washed in 70% ethanol in the field, were placed in Eppendorf tubes for two days at room temperature in the laboratory to defecate. On the third day, the fleas were taken out for further analysis, and the Eppendorf tubes with feces were washed with 100 µl of SIMB medium. The tubes with feces and SIMB medium were then vortexed, centrifuged and the final mixture was used for cultivation (Fig. 5, E).

The removed fleas were killed in 70% ethanol and washed three times in physiological saline solution. Afterward, the fleas were dissected to separate the gastrointestinal tract from the rest of the body (Fig. 6). Only the gastrointestinal tract was used for cultivation to determine if the level of culture contamination would decrease. The gastrointestinal tract was homogenized by squashing between coverslip and microscope slide and used for DNA extraction and cultivation.

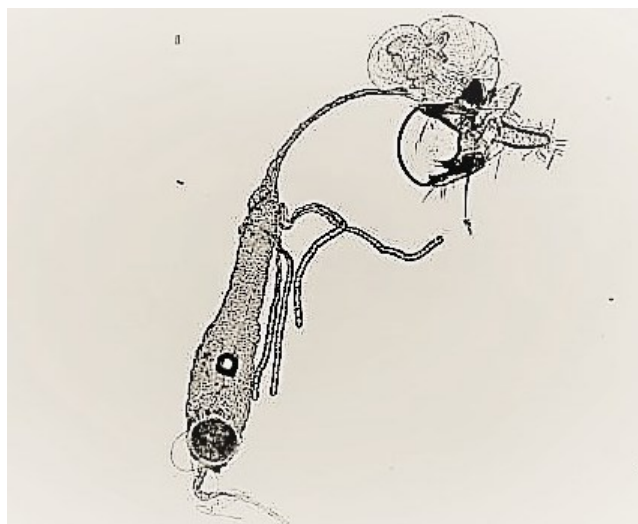


Figure 6: The digestive tract from dissected flea used for DNA extraction and cultivation. Photo by: Iveta Valsová.

### 4.3 Bartonellae cultivation

The recommendations described in Gutiérrez *et al.*, 2017 were followed for bartonellae cultivation and species determination. The samples prepared for cultivation with added 100  $\mu$ l of SIMB medium were samples of the dormice blood in EDTA, half of the homogenous mixture of fleas (removed from dormice and nests), and newly emerged adult fleas and the samples of the flea feces.

#### 4.3.1 Cultivation of samples

The mixtures of homogeneous samples in the SIMB medium were inoculated to chocolate agar plates (Tab. 1) supplied by LabMediaServis s.r.o. The samples were spread on the whole surface of the plate by a sterilized glass spreader.

Table 1: The composition of chocolate agar plates used for *Bartonella* sp. cultivation

| Ingredients                  | %    |
|------------------------------|------|
| Casein/ Animal Tissue Digest | 32.0 |
| Cornstarch                   | 2.1  |
| Sodium chloride              | 10.7 |
| Dipotassium Phosphate        | 8.5  |
| Monopotassium Phosphate      | 2.1  |
| Hemoglobin Solution          | 2.0  |
| Coenzyme enrichment          | 21.3 |
| Agar                         | 21.3 |

The agar plates were then wrapped around by sealing film (Parafilm M All-Purpose Laboratory Film) to prevent over-drying and contamination of the agar and placed inside the thermostat at 37 °C with 5% of CO<sub>2</sub> (Fig. 7). Plates were examined weekly and re-inoculated in case of other microorganism contamination. The sealing film was also changed weekly, as they start to break up after a few days thanks to the increased temperature. Individual plates were cultured for up to 6 weeks to determine their positivity or negativity for *Bartonella* spp. (Gutiérrez *et al.*, 2017).

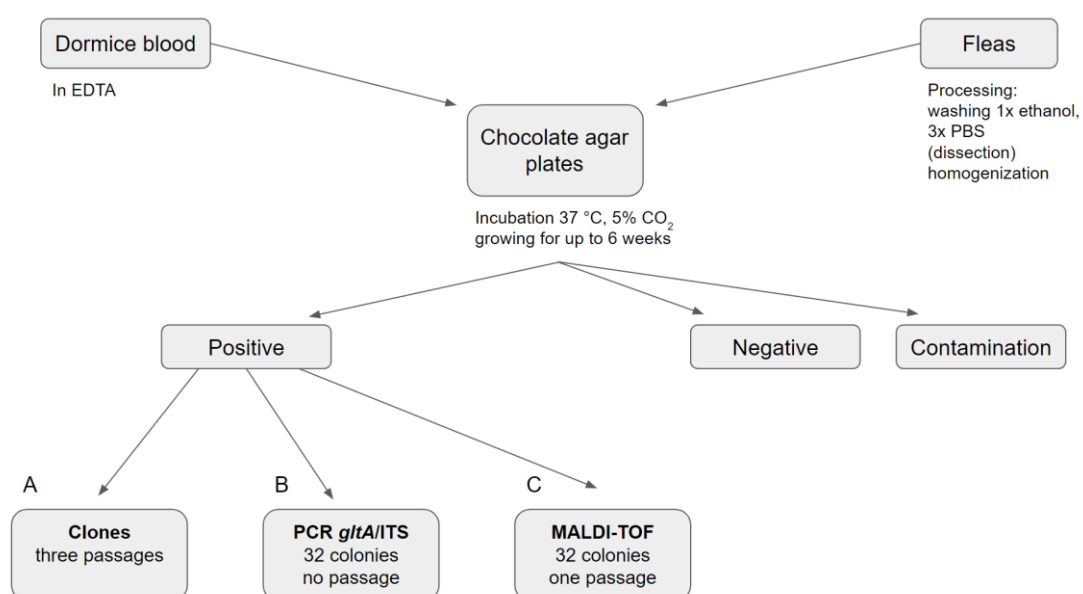


Figure 7: Scheme describing different steps of the cultivation process of samples from dormice blood and fleas.

#### 4.3.2 Cultivation of bartonellae

To obtain clones of various *Bartonella* strains, a single colony growing on the original plate was inoculated onto a new agar plate. New colonies were passaged in this way two more times on new agar plates. The culture that was passaged three times consecutively was considered to be a clone of a single *Bartonella* strain without any contamination (Fig. 7, A), as reported in previous research (Gutiérrez *et al.*, 2018). Clones of *Bartonella* spp. were identified by PCR analysis (Chapter 3.4.2.1). Cultivated *Bartonella* clones (passaged three times) were used for further analysis and characterization of genospecies.

##### 4.3.2.1 Long-term storage of cultivated samples

For the long-term preservation of *Bartonella* clones (passaged three times) of different strains, biomass was harvested from agar plates and placed in a freezing medium. The freezing medium was prepared by combining Luria-Bertani (LB) Medium and glycerol in a ratio of 4:1.

Each strain was frozen in duplicate (some of them multiple times) with 500 µl of freezing medium added to labeled 1.5 ml Eppendorf tubes. The samples were stored at –70 °C.

Some of the frozen samples were thawed after up to 12 months to check bacterial viability and to be used for further analysis. The samples were centrifuged for 2 minutes at maximum speed and the medium was discarded. The pellets were homogenized in 100 µl of SIMB medium. The pellets were again centrifuged, and the step was repeated. The homogenized suspensions were then inoculated to the chocolate agar plates and cultivated in the thermostat.

## 4.4 DNA analysis

DNA analysis was performed not only on samples from 2019 to 2021 but also on samples used by Kateřina Bundilová in her master's project, that were sampled from the years 2016 to 2018 (Bundilová, 2020). From this period, only DNA was available, therefore bacteria cultivation was not possible. The recommendations described in Gutiérrez *et al.*, 2017 were followed for *Bartonella* species identification.

### 4.4.1 *Bartonella* spp. detection by PCR

For DNA amplification, the polymerase chain reaction (PCR) was performed targeting four specific *Bartonella* sp. loci with different amplicon lengths (Tab. 2): the gene for citrate synthase (*gltA*), gene encoding beta-subunit of RNA polymerase (*rpoB*), the gene encoding a protein used in bacterial cell division (*ftsZ*), and 16S-23S rRNA internal transcribed spacer (ITS) (Birtles & Raoult, 1996; Renesto *et al.*, 2001; Maggi & Breitschwerdt, 2005; Paziewska *et al.*, 2011; Gutiérrez *et al.*, 2017).

Table 2: The length of amplicons of three different loci *gltA*, *rpoB*, and ITS used for nested PCR analysis.

| Locus       | Short amplicon size [bp] | Long amplicon size [bp] |
|-------------|--------------------------|-------------------------|
| <i>gltA</i> | 350                      | 750                     |
| <i>rpoB</i> | 400                      | 900                     |
| ITS         | 200–350                  | 900                     |

Nested PCR of the loci *gltA*, *rpoB*, and ITS was used; however, for the locus *ftsZ*, PCR was used with two steps that were the same, with the same primers and product size (515 bp). Nested PCR and PCR with repetition of the step in the case of the locus *ftsZ* were used because of the low amount of bartonellae DNA in samples and to increase the specificity and

sensitivity. The primers (Tab. 3) were prepared from the stock primers in the concentration of 5 pmol/μl of each primer.

Table 3: Primers for the loci *gltA*, *rpoB*, *ftsZ*, and ITS used for sample amplifying and sequencing.

| Locus       | PCR Type  | Primer name | Sequence 5'–3'                |
|-------------|-----------|-------------|-------------------------------|
| <i>gltA</i> | PCR 1     | 443f        | GCTATGTCTGCATTCTATCA          |
|             |           | 1210R       | GATCYTCAATCATTTCTTTCCA        |
|             | PCR 2     | 443f        | GCTATGTCTGCATTCTATCA          |
|             |           | 781R        | CCACCATGAGCTGGTCCCC           |
| <i>rpoB</i> | PCR 1     | 1400F       | CGCATTGGCTTACTTCGTATG         |
|             |           | 2300R       | GTAGACTGATTAGAACGCTG          |
|             | PCR 2     | 1596F       | CGCATTATGGTCGTATTTGTCC        |
|             |           | 2300R       | GTAGACTGATTAGAACGCTG          |
| <i>ftsZ</i> | PCR 1 & 2 | F           | CATATGGTTTTTCATTACTGCYGGTATGG |
|             |           | R           | TTCTTCGCGAATACGATTAGCAGCTTC   |
| ITS         | PCR 1     | H56s        | GGGGAACCTGTGGCTGGATCAC        |
|             |           | 983as       | TGTTCTYACAACAATGATGATG        |
|             | PCR 2     | H493as      | TGAACCTCCGACCTCACGCTTATC      |
|             |           | 321s        | AGATGATGATCCCAAGCCTTCTGG      |

For most samples, PCR reactions were performed in a total volume of 15 μl for the first step and 25 μl for the second step of PCR. For the reactions, Master Mix, PCR water, primers forward, and reverse, and isolated DNA were added in the ratio of 12:10:0.5:0.5:1. The Master Mix used in the reaction changed during the years of the project from EmeraldAmp GT PCR Master Mix to 2xDream Taq Hot Start PCR (Thermo Scientific), 2xDream Taq Hot Start Green (Thermo Scientific), and PPP Master Mix (Pop Bio). These changes did not influence the results, because before the change of the Master Mix it was first tested whether there is a difference in results using different master mixes. The Cyclers Bio-Rad T100™ Thermal Cycler and C1000 Touch™ Thermal Cycler were used for PCR with different protocols for the individual loci (Tab. 4–7). The number of cycles for ITS was changed from the original 45 to 35 in the first PCR (Tab. 7).

Table 4: The protocols for PCR of the locus *gltA* describing the type of PCR, number of cycles, temperatures for different cycles, and specific time.

| <i>gltA</i> |                  |                  |        |
|-------------|------------------|------------------|--------|
| PCR Type    | Number of cycles | Temperature [°C] | Time   |
| PCR 1       | 1x               | 95               | 5 min  |
|             | 40x              | 95               | 30 s   |
|             |                  | 53               | 30 s   |
|             |                  | 72               | 1 min  |
|             | 1x               | 72               | 10 min |
| PCR 2       | 1x               | 95               | 5 min  |
|             | 25x              | 95               | 30 s   |
|             |                  | 56               | 30 s   |
|             |                  | 72               | 1 min  |
|             | 1x               | 72               | 10 min |

Table 5: The protocols for PCR of the locus *rpoB* describing the type of PCR, number of cycles, temperatures for different cycles, and specific time.

| <i>rpoB</i> |                  |                  |       |
|-------------|------------------|------------------|-------|
| PCR Type    | Number of cycles | Temperature [°C] | Time  |
| PCR 1       | 1x               | 94               | 2 min |
|             | 35x              | 94               | 30 s  |
|             |                  | 56               | 30 s  |
|             |                  | 72               | 1 min |
|             | 1x               | 72               | 2 min |
| PCR 2       | 1x               | 94               | 5 min |
|             | 35x              | 94               | 30 s  |
|             |                  | 55               | 30 s  |
|             |                  | 72               | 1 min |
|             | 1x               | 72               | 5 min |

Table 6: The protocols for PCR of the locus *ftsZ* describing the type of PCR, number of cycles, temperatures for different cycles, and specific time.

| <i>ftsZ</i> |                  |                  |       |
|-------------|------------------|------------------|-------|
| PCR Type    | Number of cycles | Temperature [°C] | Time  |
| PCR 1 & 2   | 1x               | 94               | 5 min |
|             | 35x              | 94               | 30 s  |
|             |                  | 60               | 30 s  |
|             |                  | 72               | 1 min |
|             | 1x               | 72               | 5 min |

Table 7: The protocols for PCR of the locus ITS describing the type of PCR, number of cycles, temperatures for different cycles, and specific time.

| ITS      |                  |                  |        |
|----------|------------------|------------------|--------|
| PCR Type | Number of cycles | Temperature [°C] | Time   |
| PCR 1    | 1x               | 95               | 5 min  |
|          | 45x /35x         | 94               | 45 s   |
|          |                  | 56               | 45 s   |
|          |                  | 72               | 45 s   |
|          | 1x               | 72               | 10 min |
| PCR 2    | 1x               | 95               | 45 s   |
|          | 25x              | 94               | 45 s   |
|          |                  | 58               | 45 s   |
|          |                  | 72               | 45 s   |
|          | 1x               | 72               | 10 min |

#### 4.4.1.1 PCR of cultivated samples

##### 4.4.1.1.1 Preparation of samples

The cultured samples from the years 2020, 2021, and part of samples from 2019 were identified by direct PCR. In 2019, the protocol for a reliable description of single and mixed infections was tested and therefore first samples were determined only by analysis of a few *Bartonella* colonies (their number was not consistent) before the protocol was established.

Thereafter, from every original sample, 32 colonies were analyzed that were either passaged to new agar plates (16 colonies to one plate) for higher biomass yield (Chapter 3.5) or were analyzed directly without passaging (Fig. 7, B).

In 2020 and 2021, the colonies of cultured samples were identified only by direct PCR, and therefore lower amount of biomass for necessary<sup>2</sup>. The analysis was carried out directly on 32 colonies from the original plate. The biomass for analysis was harvested from the passaged clones or colonies from the original plate using a sterilized inoculation loop.

#### **4.4.1.1.2 PCR**

The biomass from cultivation was harvested into 1 ml microtubes with the mixture prepared for PCR reaction (Chapter 3.4.2). The species of samples were either determined by the PCR of the loci *gltA* or ITS. The analysis of *gltA* was mostly used on the samples of the clones passaged 3 times or where it was necessary to identify not only the species of the sample but also the genotype. The samples were analyzed by direct PCR due to the high amount of DNA in the samples, and therefore long products were obtained.

Most of the samples from cultivation were analyzed via PCR of the locus ITS. The short product of nested PCR visualized by electrophoresis showed different product sizes for different species and genotypes (only for one species) as opposed to the long product. To obtain long products of PCR for analysis of the detected species, the first step of PCR of the loci *rpoB*, ITS, and *gltA* was carried out on all detected species and genotypes. As the amplicon size of the locus *ftsZ* is of the same size for both steps of PCR, further analysis was not necessary.

#### **4.4.1.2 PCR protocol adjustments**

For the PCR of the loci *gltA* and *rpoB*, adjustments were tested to increase the specificity of the amplification and to obtain long products. The length of the third step of the repeated cycles for both *gltA* and *rpoB* was increased from the original 1 minute to 5 minutes.

Adjustments to PCR protocols targeting the locus ITS were tested to determine the origin of multiple bands (third band) obtained from some samples by electrophoresis. Several approaches were chosen such as the dimethyl sulfoxide (DMSO) addition to the reaction. DMSO is used to disrupt the formation of secondary structures on DNA during DNA amplification (Hardjasa et al., 2010). The effect of direct, conventional, and nested PCR on samples from cultivation and isolated DNA was compared. The number of cycles in the

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<sup>2</sup> I personally processed most of the samples from the year 2019, dormice samples from 2020 and all the samples from 2021.

protocol for the second PCR was increased from 25 to 35 and the cleaned PCR product was used for the second step of PCR again.

#### **4.4.1.3 PCR product processing**

##### **4.4.1.3.1 Electrophoresis**

The products of PCR were observed with gel electrophoresis. The gel was prepared with 1% agarose (Sigma Aldrich) in TAE (Tris-acetate-EDTA) buffer (Thermo Scientific). To observe the DNA under the UV light, SybrSafe DNA gel stain (Invitrogen) was added to a heated gel solution with dissolved agarose. The Ladder Quick Load Purple 100 bp DNA Ladder or GeneRuler 100 bp plus DNA Ladder (Thermo Scientific) was used to detect the size of DNA products on the gel. In the case of Master Mix, 2x DreamTaq Hot Start PCR (Thermo Scientific), loading dye 6x DNA Loading dye (Thermo Scientific) was used. The resulting gel was visualized under UV light and images of all gels were taken.

Products of PCR of the locus ITS were mostly not processed any further, as by electrophoresis two most common species of *Bartonella* in the samples could be distinguished by the product size. However, samples at the start of the project were also sequenced. Samples with multiple bands on the gel were cut out from the gel using a sterilized blade and put into sterilized 1.5 ml Eppendorf tubes.

Adjustments to electrophoresis protocols were tested for the products of PCR targeting the locus ITS to determine the origin of multiple bands obtained from some samples by electrophoresis. The products of PCR protocols that were adjusted were run on 2% agarose for up to 180 minutes at 110 V to enable easier cutting of separate bands from the gel. The samples with more bands were cut out from the gel using a sterilized blade and put into sterilized 1.5 ml Eppendorf tubes.

##### **4.4.1.3.2 PCR product cleanup and preparation for sequencing**

All positive samples from PCR targeting the genes *gltA*, *rpoB*, and *ftsZ* were cleaned and prepared for sequencing. From the positive samples, 5 µl of PCR product was pipetted into new tubes and 1 µl of ExoSAP-IT™ (Thermo Fisher Scientific) was added on ice. The samples were then put into a cycler used for PCR in the previous steps to heat up to 37 °C for 8 minutes then to 80 °C for 2 minutes and cool down to 4 °C.

The excised gel pieces obtained by electrophoresis of the PCR product targeting the locus ITS were weighted and the PCR product was extracted using the High Pure PCR Product Purification Kit (Roche) or E. Z. N. A. Gel Extraction Kit (Omega BIO-TEK) according to the recommended procedure.

The PCR products cleaned by either ExoSAP-IT™ or extraction from the gel were prepared for Sanger sequencing according to the sequencing laboratory instructions. In 2019, samples were sent for sequencing to the SEQme company (Dobříš), and samples were prepared by mixing 1 µl of cleaned PCR product, 8 µl of water, and 1 µl of sequencing primers. The sequencing primers were prepared from the same stock of primers as for PCR with the concentration of 3.2 pmol/µl, the individual primers for different loci were *gltA*: 443F, *rpoB*: 2300R, *ftsZ*: F, *ITS*: H493as. Later, the samples were sent to the sequencing laboratory of the Faculty of Science, Charles University in BIOCEV (Prague). The samples were prepared by mixing 1 µl of cleaned PCR product, 5.5 µl of water, and 1.5 µl of sequencing primers. If the visualized samples showed weak positive on the electrophoresis gel, 2 µl of the cleaned PCR product was added.

## 4.5 MALDI-TOF

The determination of the species of cultivated bacteria by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF) was done in cooperation with the Military Health Institute. Dormice blood and flea samples used for this analysis were from sampling in 2019 and cultivated from autumn 2019 to summer 2020. MALDI-TOF was chosen for the identification of bartonellae samples because of its effectiveness and the possibility of timesaving compared to the identification by direct PCR identification.

### 4.5.1 Samples preparation

To determine the *Bartonella* species, the colonies were passaged on new chocolate agar plates (Fig. 7, C). For MALDI-TOF analysis, single colonies could not be used due to the low amount of biomass. However, it was not necessary to work with the clones that were passaged three times in succession, and clones that were passaged only once were used. From every sample, 32 colonies were passaged, altogether 16 colonies were inoculated on an agar plate divided into 16 parts to save space. The biomass for analysis was harvested from the passaged clones using a sterilized wooden toothpick.

### 4.5.2 *Bartonella* spp. database creation

Because the Bruker Taxonomy Database did not include the *Bartonella* species that were detected on agar plates during this project, a new database was created from measurements of cultured clones (passaged three times). The biomass harvested from agar plates was extracted by ethanol and formic acid because direct colony identification was not possible due

to the low biomass yield from cultures. The biomass was harvested from each plate and added to 300 µl of water (HPLC-grade) and thoroughly mixed. 900 µl of 70% ethanol was added, and the samples were again thoroughly vortexed. Afterward, the samples were centrifuged for 2 minutes at maximum speed (Spectrafuge 16M Labnet) and the supernatant was removed. This step was repeated, and the rest of the ethanol was removed. The open tubes containing the samples were placed for 7 minutes into an incubator set at 37 °C to vaporize any leftover ethanol. To the pellet, 10 µl of 70% formic acid was added and the samples were vortexed. The same volume of 100% acetonitrile was added, and the samples were mixed carefully. The samples were centrifuged at a maximum speed for 2 minutes. 1 µl of the resulting supernatant was pipetted onto a MALDI-TOF plate and allowed to dry. 1 µl of the 4-HCCA Matrix ( $\alpha$ -Cyano-4-hydroxycinnamic acid) was added to the dried sample drop and allowed to dry for several minutes. In each run, positive control of the bacterial test standard (BTS) was added. The samples were evaluated by MALDI-TOF by Dr. Hana Kabíčková and a new library was created from the data.

#### **4.5.3 Analysis of bartonellae samples**

*Bartonella* clones were prepared for MALDI-TOF analysis using the same protocol as clones (passaged three times) for the library. The analysis was performed by H. Kabíčková on the Microflex LT Bruker, and the results were analyzed with the software flexControl, flexAnalysis, MBT Compass, and Bruker Taxonomy. The clones were examined by MALDI-TOF analysis on three different occasions from February 2020 to July 2020. From the analysis, score values that describe the validity of the identification were obtained. A score value above two is considered valid identification of species level, while an identification ranging with a value from 1.7 to 2 is considered valid identification only on the genus level. The identification of samples with a score value lower than 1.7 is not considered accurate and therefore is not valid (Wieser *et al.*, 2012; Liébana-Martos, 2018). The accuracy was determined by comparing the results of the MALDI-TOF analysis with the determination of species by sequencing or visualization of the PCR product by electrophoresis.

#### **4.6 Characterizations of detected *Bartonella* species**

The three times passaged *Bartonella* clones were used for further analysis and characterizations of the *Bartonella* species; two species, both in two genotypes detected in cultures were used.

#### **4.6.1 Transmission Electron Microscopy**

In cooperation with the Military Health Institute, transmission electron microscopy (TEM) was performed using two methods of staining and visualization.

##### **4.6.1.1 Negative staining**

Negative staining was used for bacteria visualization as it is considered a more accurate method of visualization (compared to the direct staining) because the samples are not heated up during the preparation and the stain does not enter the cells. For negative staining, formvar-coated carbon-reinforced grids (300 mesh) were used. The grids were conditioned for hydrophilicity by 1% Alcian blue in 1% acetic acid for 10 minutes. The grids were then washed by brief contact with deionized water before placing them either on a 30 µl drop of *Bartonella* sp. suspension with the grid surface or by a touch of the grid with the cultured colonies for particle absorption. Afterward, each grid was washed on four drops of deionized water in succession; the excess water was aspirated with filter paper. The grids were immediately stained by brief contact with a drop of 2% solution of phosphotungstic acid.

##### **4.6.1.2 Fixation and embedding**

The suspended cells of bartonellae were fixed with the solution of 2.5% glutaraldehyde (Polysciences) and 5 mM CaCl<sub>2</sub> in 0.1 M cacodylate buffer (pH 7.2) for two hours at room temperature. The pellets were washed three times in succession with PBS and then resuspended and centrifuged before the next step. After fixation, 0.8% potassium ferricyanide, 1% OsO<sub>4</sub>, and 5 mM CaCl<sub>2</sub> in 0.1 M cacodylate buffer were added to the samples. After 15 minutes, the samples were washed with PBS three times in succession. The dehydration of the samples was performed with a row of different acetone concentrations that were added to the samples (70%, 70%, two times 90%, three times 100%). The first acetone step was followed by 3 minutes at 13 000 rpm in RT Hettich Micro 20 centrifuge when a hard pellet was formed. Each subsequent acetone step was followed by 15 minutes of incubation before the acetone was removed and a new one was added.

After dehydration, the samples were submerged in Epon-Durcupan 1.1 diluted with 100% acetone for 30 minutes. This step was repeated once again with diluted Epon-Durcupan overnight and non-diluted Epon-Durcupan for 15 minutes three times in succession. Afterward, the samples were embedded in Epon-Durcupan; Epon-Durcupan was poured into silicone forms and the pellet was carefully placed on the tip of the form. The samples were left to polymerize for 24 hours at a temperature of 60 °C.

#### 4.6.1.3 Sample preparation for TEM

The prepared embedded samples were cut into ultrathin sections, which were placed on a copper grid and subsequently contrasted with lead citrate and uranyl acetate<sup>3</sup>. The prepared grids with *Bartonella* spp. samples, as well as the grids with negative stained samples, were visualized by a transmission electron microscope (JEM 200CX Jeol) at the Military Health Institute with the help of dr. Kabíčková. Photographs were taken with a Veleta G3 TEM camera (EMSIS) and visualized with a SW Radius (EMSIS).

#### 4.6.2 Temperature and CO<sub>2</sub> requirements

The requirements of the cultured samples for temperature and CO<sub>2</sub> level during growth were investigated. The samples of three times passaged *Bartonella* clones of all species (both in two genotypes) were used during the experiment.

Cultures were inoculated on chocolate agar plates with one inoculation loop with which three strokes by either side of the loop were made on a new agar plate. Another inoculating loop was used to spread the colonies further in additional 6 strokes. The agar plates were placed either to the thermostat with or without 5% of CO<sub>2</sub>. The thermostat without additional CO<sub>2</sub> was set to 37 °C and the thermostats with additional CO<sub>2</sub> were set to three different temperatures. A temperature of 37 °C, was recommended for bartonellae growth (Gutiérrez *et al.*, 2017) and used in this project, and two different temperatures, 32 and 42 °C were tested. Due to the limited number of available thermostats with CO<sub>2</sub>, the experiment was conducted in two parts. One thermostat was set at a temperature of 37 °C and 5% CO<sub>2</sub>, another thermostat was set at a temperature of 32 °C for the first round and 42 °C for the second, with an additional 5% CO<sub>2</sub>. The last thermostat was set at the same temperature of 37 °C in both parts of the experiment but without additional CO<sub>2</sub>. The cultures were grown for 10 days, and every second day, the cultures were examined, and photographs were taken to visualize their growth rate.

### 4.7 Analysis of the results

#### 4.7.1 Statistic data analysis

Multiple linear regression for binomial data was used with the function 'glm' to model the effect of multiple variables: age, weight, sex, and year on the detected bartonellae prevalence in individual dormice. As the sequential Chi-square test was used, the independent variables

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<sup>3</sup> I personally participated in preparation (negative staining, fixation and embedding) of samples and subsequent visualization by TEM.

were tested in various order to ensure the robustness of the results. The binomial generalized linear mixed model with the function 'glmer' was used to model the differences in the prevalence of three flea's life stages: (i) primary adult fleas which can be blood-fed on dormice, (ii) larvae, and (iii) newly emerged adult fleas with no contact with dormice. The variable '*Bartonella* spp. presence/absence' in individual fleas was tested by type II Wald Chi-square test with the fixed effect of the life stage of fleas and the random effect of the nest identity. The package 'lme4', 'ggplot2', 'grid', and 'ggpubr' were used for generalized linear models and all data visualization (Bates *et al.*, 2015; Wickham, 2016; Kassambara, 2020; R Core Team, 2022) using the statistical computing environment R Studio 2022.02.0 Build 443 (RStudio Team, 2022).

#### 4.7.2 Sequence analysis

All manipulation of the obtained sequences (including alignment creation and phylogenetic analysis) took place in a Geneious Prime environment (Geneious Prime 2022.1.1, 2022). The sequences were adjusted (end trimming, unclear nucleotide reading correction, etc.) and the resulting sequences were aligned to form consensus sequences for all detected variants. The consensus sequences of all detected variants were compared to other *Bartonella* spp. sequences in National Centrum for Biotechnology Information (NCBI) GenBank databases using BLASTn analysis (<https://www.ncbi.nlm.nih.gov>).

Consensus sequences of all four loci (long versions in the case of nested PCRs) obtained from three times passaged clones were concatenated and aligned with sequences of *Bartonella* spp. for which all four loci were available. The phylogenetic relationships of the concatenated sequences were analyzed in Geneious Prime using Bayesian inference (BI; MrBayes; GTR model (nst=6), invgamma (4 categories), MCMC 5,500,000 generations, subsampling frequency 200, burn-in length 550,000) and maximum likelihood method (ML; PhyML; GTR model (nst=4) selected based on jModelTest (Posada, 2008) with the bootstrap value of 1000). PhyML and Bayesian inference analysis (with the same parameters as described above) were used for phylogenetic tree construction of the concatenated amino acids sequences (derived from *gltA*, *rpoB*, and *ftsZ*). Additionally, short versions of all consensus sequences for *gltA* and short versions of the consensus sequence of one species for *rpoB* obtained directly from hosts by nested PCR were aligned with reference sequences and used for two separate phylogenetic analyses via PhyML as described above.

## 5 Results

### 5.1 Bartonellae in dormice blood and samples of fleas

#### 5.1.1 Bartonellae in dormice blood samples

A total of 204 blood samples were obtained from the dormice that were investigated from 2016 to 2021. The blood samples originated from 203 individuals as only one dormouse was sampled repeatedly (based on a veterinary microchip; in 2019 and 2020), and in both cases, it was positive for *Bartonella* spp., and mixed infections were detected.

In total, 68 females (33%) and 127 (63%) males were sampled (Fig. 8) and for 8 dormice the sex was not determined. From a total of 203 sampled dormice, 71 (35%) were determined as juveniles (before the first hibernation), 87 (43%) adults and for 45 dormice the age was not determined (22%). The lack of information on the age and sex of the dormouse was in most cases in the samples obtained from Bundilová. The weight of altogether 202 individuals was measured and ranged from 23 to 190 grams (Fig. 8). Multiple regression was used to determine the influence of multiple variables (age, weight, sex, year) on *Bartonella* spp. prevalence in dormice samples. Only the dormice age was described to have a significant positive effect ( $p < 0.01$ , for all combinations) on *Bartonella* spp. prevalence; (79%) juveniles and 82 (93%) adults were found *Bartonella*-positive.

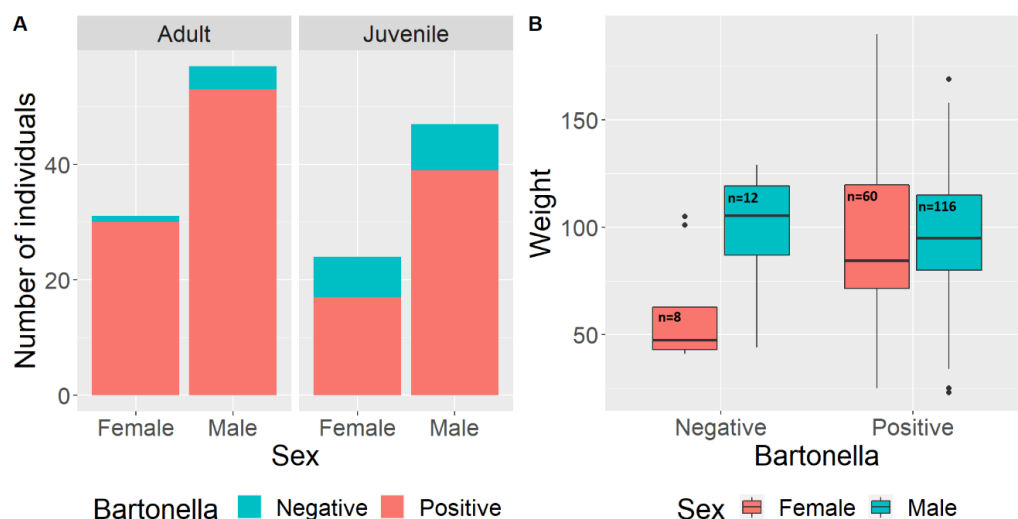


Figure 8: A: *Bartonella* sp. positivity according to sex and age of sampled dormice. B: Dormice detected to be positive/negative on *Bartonella* sp. dependent on their sex and weight.

Since the year 2021 was poor in the number of seeds in the forests, there were not many dormice in the nest boxes and only three individuals were sampled. In the last three seasons (from 2019 to 2021), blood samples from a total of 81 individuals were used for

cultivation (Tab. 8). Fewer samples were used for cultivation than for DNA extraction, as not enough blood was sampled from five dormice (four from 2019 and one from 2020). *Bartonella* sp. was detected by PCR in 180 out of 204 blood samples from dormice (88%) and 49 out of 81 cultivated blood samples (61%); the total combined prevalence was 89%.

Table 8: Number of blood samples taken from dormice between 2016 and 2021 and tested by PCR (for 4 loci) and cultured on agar plates (incl. contaminated samples).

| Year    | PCR              |              | Cultivation      |              |                   |
|---------|------------------|--------------|------------------|--------------|-------------------|
|         | Positive/Overall | Positive [%] | Positive/Overall | Positive [%] | Contamination [%] |
| 2016    | 14/16            | 88           | 0                |              |                   |
| 2017    | 55/62            | 90           | 0                |              |                   |
| 2018    | 38/40            | 95           | 0                |              |                   |
| 2019    | 52/63            | 83           | 36/59            | 61           | 25                |
| 2020    | 17/20            | 85           | 12/19            | 63           | 26                |
| 2021    | 3/3              | 100          | 1/3              | 33           | 67                |
| Overall | 180/204          | 88           | 49/81            | 60           | 27                |

## 5.1.2 Fleas

### 5.1.2.1 Flea species determination

Altogether, 823 fleas were used for bartonellae detection: 510 originated from dormice and 313 from their nests. Primary adult fleas and newly emerged adult fleas from the dormice nest experiment were also included. Altogether, 204 fleas were identified by Bundilová as *Ceratophyllus (Monopsyllus) sciurorum* (Bundilová, 2020), and their extracted DNA was used for further analysis. Since the vast majority of fleas associated with dormice were detected as *C. (Monopsyllus) sciurorum*, species determination under the stereomicroscope was not performed on all samples.

The species and sex were determined for a total of 410 fleas (Tab. 9), with females making up 60% of all fleas examined. Almost all (92%) fleas were detected as *Bartonella*-positive, only 21 females and 10 males were negative, and the prevalence did not differ between the sexes.

Table 9: Rates of bartonellae infections in 410 fleas (all determined as *Ceratophyllus (Monopsyllus) sciurorum*) according to their sex.

| Sex/ <i>Bartonella</i> | Positive | Negative |
|------------------------|----------|----------|
| Males                  | 94       | 6        |
| Females                | 91       | 9        |
| Total                  | 92       | 8        |

#### 5.1.2.2 Bartonellae in fleas collected directly from dormice

In 2016, no fleas were sampled. Flea samples from seasons 2017 and 2018 were received in the form of extracted DNA (Bundilová, 2020). Only fleas from 2019 to 2021 could be used for cultivation. A total of 510 flea DNA samples (Tab. 10) were analyzed and *Bartonella* sp. was detected by PCR (four loci) in 458 fleas (90%). Of 79 flea samples tested on blood agar plates, only 17 (22%) were positive for bartonellae (however, 34 (43%) samples were contaminated and therefore the positivity could not be assessed). The overall combined positivity determined by PCR and cultivation was established to be 90%.

Table 10: Number of fleas collected directly from dormice during five breeding seasons (2017 to 2021) and tested by PCR (for 4 loci) and cultured on agar plates (incl. contaminated samples).

| Year    | PCR              |              | Cultivation      |              |                   |
|---------|------------------|--------------|------------------|--------------|-------------------|
|         | Positive/Overall | Positive [%] | Positive/Overall | Positive [%] | Contamination [%] |
| 2017    | 100/102          | 98           |                  |              |                   |
| 2018    | 91/102           | 89           |                  |              |                   |
| 2019    | 197/222          | 89           | 8/31             | 26           | 35                |
| 2020    | 29/35            | 83           | 6/18             | 33           | 33                |
| 2021    | 41/49            | 84           | 3/30             | 10           | 57                |
| Overall | 458/510          | 90           | 17/79            | 22           | 43                |

#### 5.1.2.3 Bartonellae in fleas collected from dormice nests

In the years 2016 to 2018, no fleas were sampled from nests. A total of 155 adult fleas collected from nests between 2019 and 2021 were analyzed by PCR of four loci (Tab. 11) and *Bartonella* sp. was detected in 94 fleas (39%). Some fleas (43 specimens) were also used for cultivation, but only 3 (7%) were positive for bartonellae (however, 21 (49%) samples were contaminated). Overall, the positivity of fleas collected from nests and determined by PCR and cultivation was 40%.

Table 11: Number of fleas collected from dormice nests during three breeding seasons (2019 to 2021) and tested by PCR (for 4 loci) and cultured on agar plates (incl. contaminated samples).

| Year    | PCR              |              | Cultivation      |              |                   |
|---------|------------------|--------------|------------------|--------------|-------------------|
|         | Positive/Overall | Positive [%] | Positive/Overall | Positive [%] | Contamination [%] |
| 2019    | 3/10             | 30           |                  |              |                   |
| 2020    | 28/65            | 43           | 1/10             | 10           | 80                |
| 2021    | 30/112           | 38           | 2/33             | 6            | 39                |
| Overall | 62/155           | 40           | 3/43             | 7            | 49                |

#### 5.1.2.3.1 Occurrence of bartonellae in different flea life stages

The experiment with *Bartonella* sp. transstadial transmission and its occurrence at various flea stages took place in 2019 and 2021. Because the experiment in 2019 was a pilot one, the experiment was used mainly to test methods and manipulations with nests. The nests were sampled at the end of winter to ensure the absence of adult fleas. However, in the fleas removed from nests the presence of *Trypanosoma* sp. was detected (as part of a different study) that could be obtained only via feeding on the host (Strickland & Swellengrebel, 2009), indicating that some of the fleas were primary adults instead of only newly emerged adult fleas.

The experiment was repeated in 2021, a total of 17 nests were processed, of which 14 nests were collected from the field sampling in July and another three nests from the sampling in August. The nests were consecutively processed for half a year. Three nests had no fleas at all, and two nests had no newly emerged adult fleas and larvae; these five nests were excluded from the analysis. After transport from the field, all primary adult fleas were removed from nests, but only a maximum of 10 fleas per nest were further processed (except for the nest number H15 with 22 fleas) (Tab. 12). Up to 10 larvae and 25 newly emerged adult fleas were collected and processed per nest.

Table 12: Numbers of tested and ITS-PCR *Bartonella*-positive primary adult fleas, larvae, and newly emerged adult fleas from dormice nests.

| Nest    | Primary adult fleas |              | Larvae       |              | Newly emerged adult |          |
|---------|---------------------|--------------|--------------|--------------|---------------------|----------|
|         | Positive/Overall    | Positive [%] | Pos./Overall | Positive [%] | Pos./Overall        | Positive |
| H3      | 1/10                | 10           | 0/3          | 0            | 4/25                | 16       |
| H4      | 2/10                | 20           | 2/7          | 29           | 3/4                 | 75       |
| H6      | 5/10                | 50           | 6/10         | 60           | 2/11                | 18       |
| H8      | 1/10                | 10           | 0/8          | 0            | 0/10                | 0        |
| H9      | 8/11                | 73           | –            | –            | 0/4                 | 0        |
| H9b     | 0/3                 | 0            | 0/4          | 0            | –                   | –        |
| H10     | 2/10                | 20           | 9/10         | 90           | 4/11                | 36       |
| H11     | 0/10                | 0            | 3/4          | 75           | 5/10                | 50       |
| H12 D   | 5/6                 | 83           | 0/7          | 0            | 1/10                | 10       |
| H12 H   | –                   | –            | –            | –            | 0/10                | 0        |
| H13     | 1/4                 | 25           | 1/10         | 10           | 3/10                | 30       |
| H15     | 8/22                | 30           | 1/10         | 10           | 0/21                | 0        |
| Overall | 33/106              | 30           | 22/73        | 30           | 22/126              | 19       |

A total of 31% (33/106) of primary adult fleas, 30% (22/73) of larvae, and 17% (22/126) of newly emerged adult fleas were detected to be *Bartonella*-positive by ITS-PCR. However, the model showed (Fig. 9) that there was no significant difference ( $p = 0.26$ ) in bartonellae prevalence between the three stages tested.

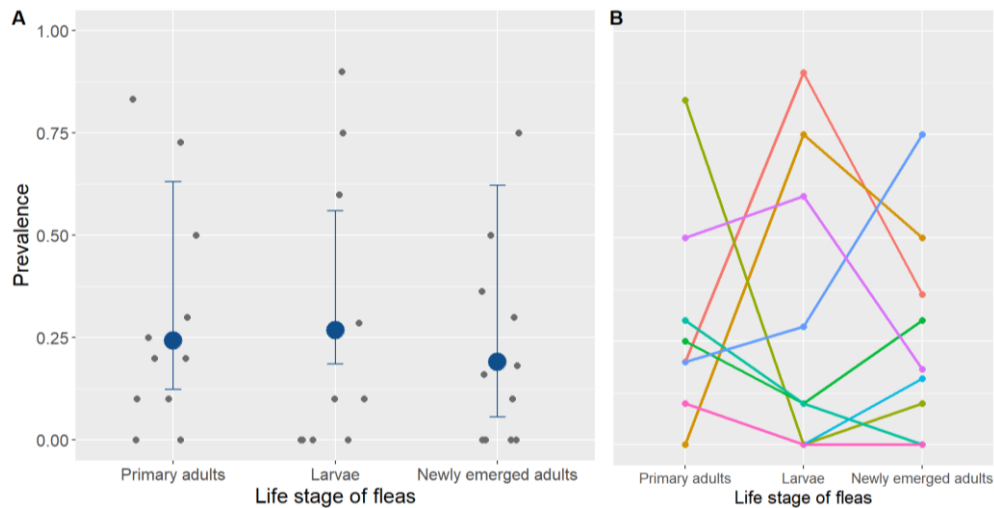


Figure 9: Bartonellae prevalence in three different life stages of fleas (primary adults, larvae, and newly emerged fleas). A) The estimated prevalence of fleas in different life stages across nests is visualized by blue dots with 95% confidential intervals. The values of the *Bartonella* sp. prevalence of fleas in different life stages are shown for each nest separately (black dots). B) Comparison of *Bartonella* sp. prevalence in fleas in different life stages from nests (only the values of nests with all flea life stages are visualized).

#### 5.1.2.3.2 Bartonellae in flea feces

Of the 49 fleas washed with ethanol, 17 died later in the Eppendorf tubes (probably due to washing). Of the 32 fleas that survived, only 26 defecated; all 26 samples of feces were cultivated but were overgrown by contamination (and therefore could not be analyzed).

### 5.2 *Bartonella* species determination

*Bartonella* species were determined by sequencing four different PCR products (*gltA*, *rpoB*, *ftsZ*, and in some cases ITS); however, the results of ITS-PCR were evaluated primarily by visualization by electrophoresis. Altogether, four different *Bartonella* species were identified; however, two species, *Bartonella washoensis* and '*Candidatus Bartonella rudakovii*', were recognized only in one sample each and not via all loci. The remaining two species were common in dormice and flea samples. One was determined as a new subspecies of *Bartonella grahamii* and is designated in this study as *B. grahamii*-like, and the other represents a novel species of *Bartonella* (designated in this study as *Bartonella* sp. NEW). Furthermore, several distinct genotypes were detected for both *B. grahamii*-like and *B. sp. NEW*, differing by one or more nucleotide substitutions at the three loci tested: 4 and 4 in *gltA*, 3, and 3 in *rpoB*, and 3 and 2 in ITS, respectively.

#### 5.2.1 Citrate synthase gene (*gltA*)

Through the analysis of the *gltA* sequences, four *Bartonella* species were determined. *B. washoensis* and '*C. B. rudakovii*' were recognized in only one sample each. *B. washoensis* was identified in a sample of a flea removed from a dormouse in 2019 (and confirmed in the same sample by *rpoB*, *ftsZ*, and ITS sequence analysis). '*C. B. rudakovii*' was found in a flea removed from a nest in 2021, but unlike *B. washoensis*, the loci *rpoB*, *ftsZ*, and ITS did not amplify by PCR.

Using short sequences from nested PCR, the species *B. grahamii*-like and *B. sp. NEW* were recognized in several different genotypes: *B. grahamii*-like in three (1, 3, 4) and *B. sp. NEW* in four (1–4) genotypes. The long products were successfully obtained from cultures only, due to the lower sensitivity of the first step of PCR. However, the analysis of the long products showed several additional nucleotide substitutions and revealed one more genotype (described here as *B. grahamii*-like 2), which was not distinguishable from *B. grahamii*-like 1 in the short product sequence (Fig. 10). On the other hand, *B. grahamii*-like 2 is more similar to *B. grahamii*-like 3 by analysis of long products. All four genotypes of

*B. sp. NEW* differed from each other by one or two nucleotide substitutions visible in short products, and the analysis of long products did not discover any additional substitutions.

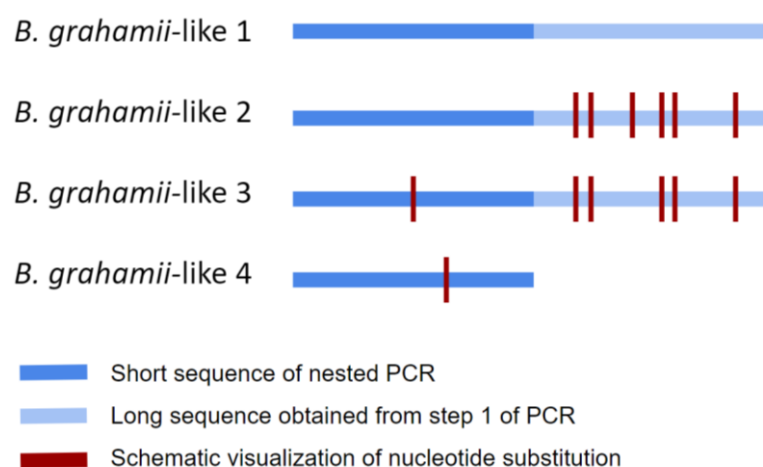


Figure 10: Scheme of sequences of *B. grahamii*-like genotypes (1–4) obtained from PCR targeting *gltA* with both short (nested PCR) and long (one-step PCR) products; for *B. grahamii*-like genotype 4 only a short product is available.

Only two genotypes of the species *B. grahamii*-like (1/2, 3) and *B. sp. NEW* (1, 2) were common in the samples (note that genotypes *B. grahamii*-like 1 and 2 could not be distinguished by short product); the other genotypes were observed just once. *B. grahamii* 4 and *B. sp. NEW* 3 were detected in dormice blood samples from 2017. *B. sp. NEW* 4 was determined in a flea that was removed from nests in 2020. None of these three rare genotypes was recognized as a separate genotype at any other loci analyzed (Tab. 13).

Table 13: The origin of rare genotypes recognized by *gltA* sequencing and sequencing results of other loci.

| Organism | Year | <i>gltA</i>                | <i>rpoB</i>  | <i>ftsZ</i>              | ITS               | Cultivation |
|----------|------|----------------------------|--------------|--------------------------|-------------------|-------------|
| Dormice  | 2017 | <i>B. grahamii</i> -like 4 | bad sequence | negative                 | negative          |             |
| Dormice  | 2017 | <i>B. sp. NEW</i> 3        | bad sequence | <i>B. grahamii</i> -like | <i>B. sp. NEW</i> |             |
| Flea     | 2020 | <i>B. sp. NEW</i> 4        | negative     | negative                 | negative          | negative    |

### 5.2.2 RNA polymerase beta-subunit gene (*rpoB*)

Three different species were identified by *rpoB* analysis. While *B. washoensis* was detected in a single flea (Chapter 4.2.1), more abundant *B. grahamii*-like and *B. sp. NEW* were recognized in several genotypes. Using the short product of nested PCR, the species *B. grahamii*-like and *B. sp. NEW* were recognized in 2 and 3 genotypes, respectively. All observed genotypes of

*B. grahamii*-like and *B. sp. NEW* differed from each other by single nucleotide substitution. The long products were successfully obtained from cultures only (*B. grahamii* 1–3, *B. sp. NEW* 1, 2 recognized by *gltA*) and a comparison of detected genotypes across loci was performed (Chapter 4.2.5). The analysis of long products of *rpoB* did not recognize any additional substitutions between *B. grahamii*-like 1 and 2 (Fig. 11). However, the analysis revealed the presence of an additional genotype, differing by a single nucleotide substitution in the long product; however, the short product was identical to *B. grahamii*-like 1. This genotype is referred to as *B. grahamii*-like 3. It became clear from cultures characterization, that genotypes *B. sp. NEW* 1 and 2 recognized by *gltA* do not differ in *rpoB* sequence and are both recognized as *B. sp. NEW* 1/2. Because samples of the rare genotypes (*B. sp. NEW* 5, 6) were not cultured, the long products of *rpoB* are not available due to the lower sensitivity of primers.

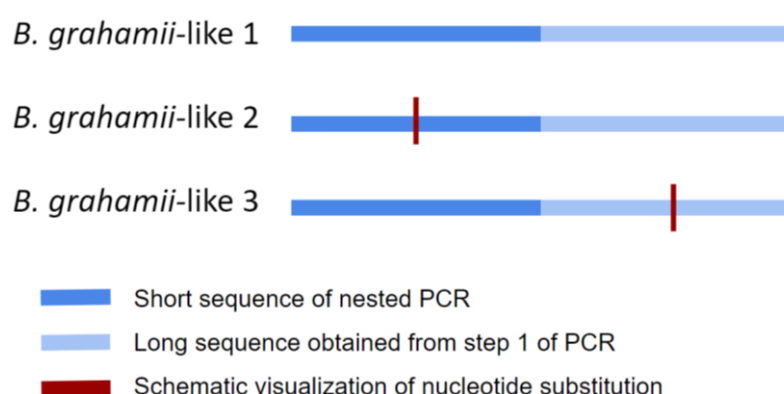


Figure 11: Scheme of sequences of *B. grahamii*-like genotypes (1–3) obtained from PCR targeting *rpoB* with both short (nested PCR) and long (one-step PCR) products.

While two *B. grahamii*-like genotypes (recognized by short product) were common among the examined samples, out of the three *B. sp. NEW* genotypes only one genotype was common in the analyzed samples. Both *B. sp. NEW* 5 and 6 (more likely different from the genotypes 3 and 4 by *gltA* because they were found in different samples) were each found in a single flea removed from the dormice in 2020. For these two samples, PCR amplification was unsuccessful for all other loci (Tab. 14).

Table 14: The origin of rare genotypes recognized by *rpoB* sequencing and PCR results of other loci.

| Organism | Year | <i>gltA</i> | <i>rpoB</i>         | <i>ftsZ</i> | ITS      |
|----------|------|-------------|---------------------|-------------|----------|
| Flea     | 2020 | negative    | <i>B. sp. NEW</i> 5 | negative    | negative |
| Flea     | 2020 | negative    | <i>B. sp. NEW</i> 6 | negative    | negative |

### 5.2.3 Gene for cell division protein (*ftsZ*)

The analysis of the *ftsZ* gene sequences determined three different species; the species *B. washoensis* was detected in only one sample (Chapter 4.2.1). The other two species, *B. grahamii*-like, and *B. sp. NEW* were recognized in a single genotype.

### 5.2.4 Internal transcribed spacer (ITS)

According to the ITS sequences analysis, three different species were identified. While *B. washoensis* was detected in a single flea (Chapter 4.2.1), *B. grahamii*-like and *B. sp. NEW* were recognized in more samples. Moreover, the two abundant species were distinguishable by different sizes of nested PCR amplicons by electrophoresis visualization (Fig. 12). Two genotypes (by short products) of *B. grahamii*-like differed by insertion/deletion of 26 bp and the size variation also enabled their identification without sequencing (Fig. 12, A). Only one genotype of *B. sp. NEW* was detected by the short products.

In addition, a third band (Fig. 12, B) was recognized via electrophoresis visualization and the possibility of the presence of another genotype was tested by adjusted PCR analysis (Chapter 3.4.2.2). Although different adjustments of the ITS-PCR protocol were tested, the visualization of samples by electrophoresis did not show any improvement in the PCR specificity; the origin of the additional band was not clarified, and it remains unknown whether the third band was just an artifact or an additional genotype.

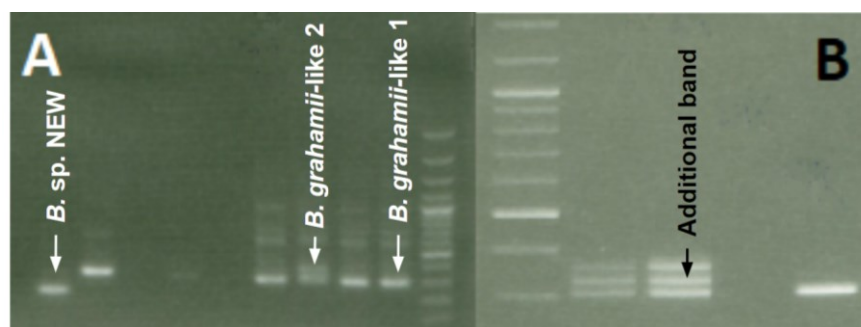


Figure 12: PCR products of the ITS locus visualized by electrophoresis, A: showing different sizes of the PCR products for *B. sp. NEW* (217 bp), *B. grahamii*-like 1 (311 bp), and mixed infections of *B. grahamii*-like 1 and *B. grahamii*-like 2 (337 bp). B: showing the third band of a size similar to *B. grahamii*-like.

The long products were successfully obtained from cultures only (*B. grahamii*-like 1–3, *B. sp. NEW* 1, 2 recognized by *gltA*) and a comparison of detected genotypes across loci was performed (Chapter 4.2.5). The analysis of the long products showed that there were no additional substitutions between *B. grahamii*-like 1 and *B. grahamii*-like 2 (Fig. 13), on the other hand, a deletion was observed in *B. grahamii*-like 2 (21 bp). Furthermore, the analysis of

*B. grahamii*-like 2 long products revealed the presence of an additional genotype that lacked a deletion recognized in *B. grahamii*-like 2 long products (Fig. 13). The newly detected genotype is referred to as *B. grahamii*-like 3 and is identical to *B. grahamii*-like 2 in the short product. The long ITS product of *B. sp. NEW* revealed the presence of two genotypes, differing by a single nucleotide substitution and a 22 bp long deletion in *B. sp. NEW* 1.

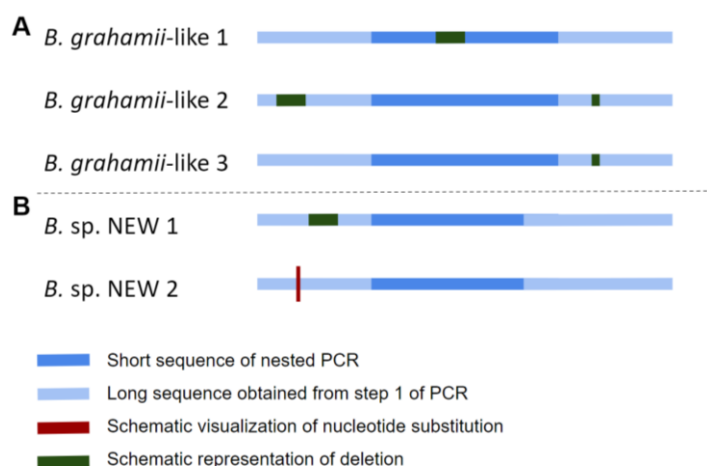


Figure 13: Scheme of sequences of A: *B. grahamii*-like genotypes (1, 2, 3) and B: *B. sp. NEW* (1, 2) obtained from PCR targeting ITS with both short (nested PCR) and long (one-step PCR) products comparing the differences between genotypes.

### 5.2.5 Genotypes characterization

The long sequences of the four analyzed loci were obtained using passaged clones of five genotypes representing two species: *B. grahamii*-like (genotypes 1, 2, and 3) and *B. sp. NEW* (genotypes 1 and 2). Thanks to the analysis of passaged clones, all cultured genotypes could be characterized by all four loci (Tab. 15). The results of genotype characterizations enabled the identification of genotypes across loci.

For the description of *Bartonella* spp. diversity in blood and flea samples, PCR analysis of four loci was used. As the nested PCR was used for all samples except for cultures, only short PCR products were obtained. However, some of the genotypes were not possible to distinguish from each other by short products (*gltA*: *B. grahamii*-like 1/2, *rpoB*: *B. grahamii*-like 1/3, ITS: *B. grahamii*-like 2/3) (Tab. 15) and therefore the identification of *B. grahamii*-like genotypes was unreliable. For this reason, only unambiguous genotypes (*gltA*: *B. grahamii*-like 3, *rpoB*: *B. grahamii*-like 2, ITS: *B. grahamii*-like 1) were counted, all the other identified genotypes were classified as unidentified genotypes.

Table 15: The results of *gltA*, *rpoB*, *ftsZ*, and ITS sequencing of three times passaged clones used for *Bartonella* genotypes characterizations.

| Description                | Short products |             |             |     | Long products |             |             |     |
|----------------------------|----------------|-------------|-------------|-----|---------------|-------------|-------------|-----|
|                            | <i>gltA</i>    | <i>rpoB</i> | <i>ftsZ</i> | ITS | <i>gltA</i>   | <i>rpoB</i> | <i>ftsZ</i> | ITS |
| <i>B. grahamii</i> -like 1 | 1/2            | 1/3         | 1/2/3       | 1   | 1             | 1           | 1/2/3       | 1   |
| <i>B. grahamii</i> -like 2 | 1/2            | 2           | 1/2/3       | 2/3 | 2             | 2           | 1/2/3       | 2   |
| <i>B. grahamii</i> -like 3 | 3              | 1/3         | 1/2/3       | 2/3 | 3             | 3           | 1/2/3       | 3   |
| <i>B. sp. NEW</i> 1        | 1              | 1/2         | 1/2         | 1/2 | 1             | 1/2         | 1/2         | 1   |
| <i>B. sp. NEW</i> 2        | 2              | 1/2         | 1/2         | 1/2 | 2             | 1/2         | 1/2         | 2   |

## 5.2.6 PCR analyses

### 5.2.6.1 Infection rates

A total of 869 DNA samples (204 dormice blood, 510 fleas collected from dormice, and 155 fleas sampled from dormice nests) were screened for bartonellae presence using a set of four different PCR reactions targeting various loci (Fig. 14).

The most sensitive assay was ITS (PCR product in 72% of samples), followed by *gltA* (69%), *rpoB* (64%), and *ftsZ* (51%). However, each of the chosen PCR protocols revealed positivity in additional samples or mixed infections of *Bartonella* species that would be missed by all other protocols. According to the hosts, the overall bartonellae prevalence was 89%, 90%, and 40% for dormice, fleas from dormice, and nests, respectively (Fig. 14).

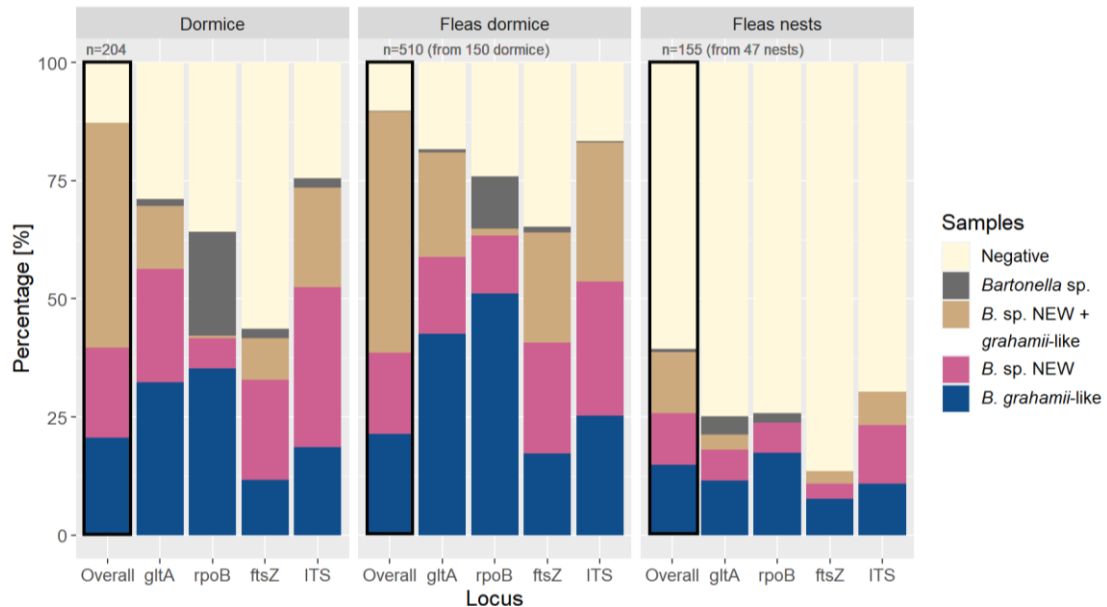


Figure 14: Comparison of *gltA*, *rpoB*, *ftsZ*, and ITS PCR sensitivity, *Bartonella* spp. infection rates, and species determination. The samples were divided into three groups according to hosts: dormice (204 individuals), fleas from dormice (510 fleas sampled from 150 dormice individuals), and fleas from nests (155 fleas from 47 nests). Rare detected species (*B. washoensis*, '*C. B. rudakovii*') together with samples, where proper species identification was not possible due to the low sequence quality are labeled as *Bartonella* sp.

The prevalence of *B. grahamii*-like single infection was detected to be identical (21%) in dormice and fleas from dormice and the prevalence of *B. sp. NEW* was detected to be similar in dormice and fleas from dormice at 19% and 17%, respectively. On the other hand, the prevalence of single infections in fleas from nests was detected to be slightly different: *B. grahamii*-like 15% and *B. sp. NEW* 11%.

Mixed infections of the two most common species, *B. grahamii*-like, and *B. sp. NEW* were found in a high proportion in dormice (48%) and fleas from dormice (51%), and in a lower proportion in fleas from nests (13%). Overall, mixed infections were revealed either by the results of one PCR locus results (clear double peaks) or by a combination of different loci PCR results. The overall results of different loci PCR showed mixed infection in 44% compared to the prevalence of mixed infection recognized by *gltA*, *rpoB*, *ftsZ*, and ITS separately as 17%, 1%, 16%, and 23%, respectively.

A total of four and six genotypes were recognized for *B. grahamii*-like and *B. sp. NEW*, respectively (Tab. 16). Three genotypes of *B. grahamii*-like were common and detected in both blood of dormice and fleas, whereas only two genotypes of *B. sp. NEW* were common. The species '*C. B. rudakovii*' and *B. washoensis* were detected only in one genotype both in a single flea.

Table 16: Genotypes of detected *Bartonella* spp. recognized in samples of dormice blood and fleas.

| <i>Bartonella</i> spp./origin | Dormice    | Fleas            |
|-------------------------------|------------|------------------|
| <i>B. grahamii</i> -like      | 1, 2, 3, 4 | 1, 2, 3          |
| <i>B. sp. NEW</i>             | 1, 2       | 1, 2, 3, 4, 5, 6 |
| ' <i>C. B. rudakovii</i> '    | 0          | 1                |
| <i>B. washoensis</i>          | 0          | 1                |

#### 5.2.6.2 Relative proportion of *B. grahamii*-like and *B. sp. NEW*

The overall proportion (in *Bartonella*-positive samples) of *B. grahamii*-like was detected to be 58% and of *B. sp. NEW* 42% (including mixed infections), showing a slightly higher proportion of *B. grahamii*-like infections in the samples (Fig. 15). A similar trend was described for samples of each host group separately, the proportion of *B. grahamii*-like in positive samples reached 56%, 59%, and 61% in dormice, fleas from dormice, and nests, respectively. However, the higher proportion of *B. grahamii*-like compared to *B. sp. NEW* in samples was caused by a higher recognition rate of genotypes of *B. grahamii*-like, because mixed infections were counted as all genotypes detected in samples. Most samples (18%) that were determined as

*Bartonella*-positive, but their sequence did not enable proper species determination, were from the *rpoB* PCR assay.

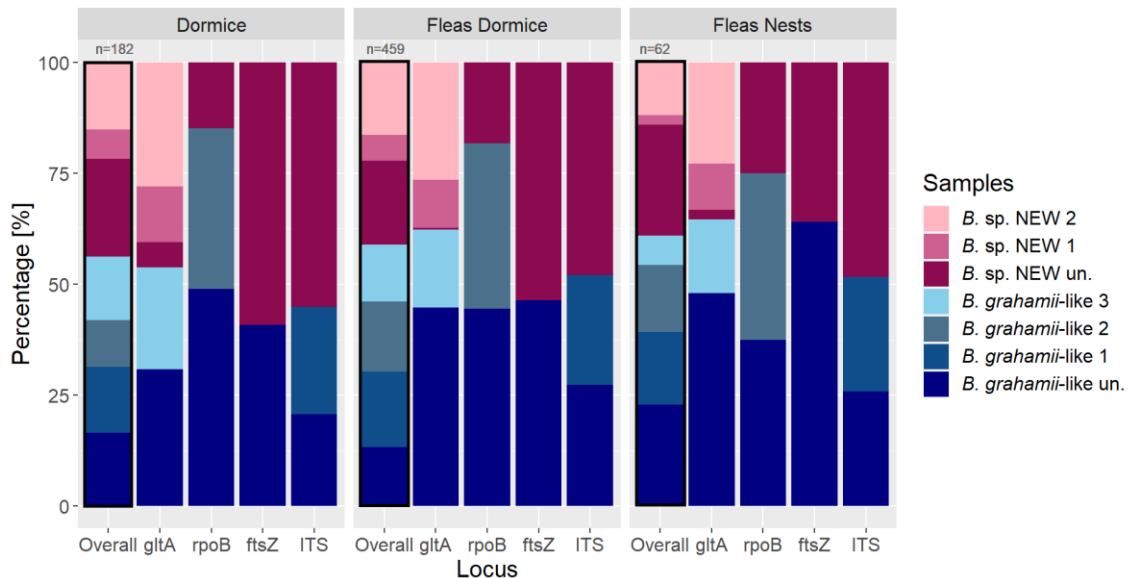


Figure 15: Comparison of the four PCR assays (*gltA*, *rpoB*, *ftsZ*, and ITS) and the relative proportion of detected species/genotypes (by short product) in *Bartonella*-positive well-sequenced samples. The samples are divided into three groups according to their origin: dormice, fleas from dormice, and fleas from nests, and the overall results of the analysis of all four loci are included. Samples with mixed infections are counted as all the species detected in the samples and unidentified genotypes are marked as un.

### 5.2.6.3 Specificity of PCR assays to detect *Bartonella* species

The results of the PCR analysis of various loci showed the specificity of different primers to the detected species and the ability to recognize mixed infections (Tab. 17). Altogether 697 samples examined by four loci were found positive. The highest specificity to *Bartonella* sp. infections showed the locus ITS that recognized 90% of positive samples. On the other hand, the lowest specificity was described by the locus *ftsZ* which recognized 64% of positive samples.

Infection of *B. grahamii*-like was detected in a total of 552 samples (as a single or mixed infection by the combination of all four loci). The highest sensitivity to *B. grahamii*-like showed the locus *gltA* that detected the species in 84% *B. grahamii*-like positive samples (Tab. 17). Infection of *B. sp. NEW* was overall detected in 522 samples and the highest sensitivity showed the locus ITS, recognizing the species in 84% of *B. sp. NEW* positive samples. Mixed infections were detected by both multiple loci and single locus in 377 samples. The highest recognition rate of mixed infections by a single locus was described for ITS (54%).

Table 17: The rate of positive samples, infections of different species, and their combination recognized by various loci (*gltA*, *rpoB*, *ftsZ*, and ITS) in samples detected to be *B. grahamii*-like and *B. sp. NEW* positive, with mixed infections and as *Bartonella*-positive.

| [%]         | <i>B. grahamii</i> -like | <i>B. sp. NEW</i> | Mixed infections | Positive |
|-------------|--------------------------|-------------------|------------------|----------|
| <i>gltA</i> | 84                       | 55                | 38               | 86       |
| <i>rpoB</i> | 67                       | 18                | 2                | 79       |
| <i>ftsZ</i> | 48                       | 59                | 37               | 64       |
| ITS         | 70                       | 84                | 54               | 90       |

### 5.2.7 Change in the prevalence of bartonellae over time

A total of 869 dormice and flea (from dormice and nests) sampled over six years were examined by complementary analyses of 4 different PCR assays and cultivation. The overall prevalence in samples was detected to be 81% and it varied over the years. Focusing on dormice, the *Bartonella* spp. prevalence ranged from 81% in 2016 to 100% in 2021, but the high prevalence observed in 2021 is questionable due to the low number of captured individuals (Fig. 16). Differences in *Bartonella* spp. prevalence were observed between fleas sampled from dormice individuals (varying from 83% in 2020 to 98% in 2017) and from their nests (varying from 38% in 2021 to 43% in 2020).

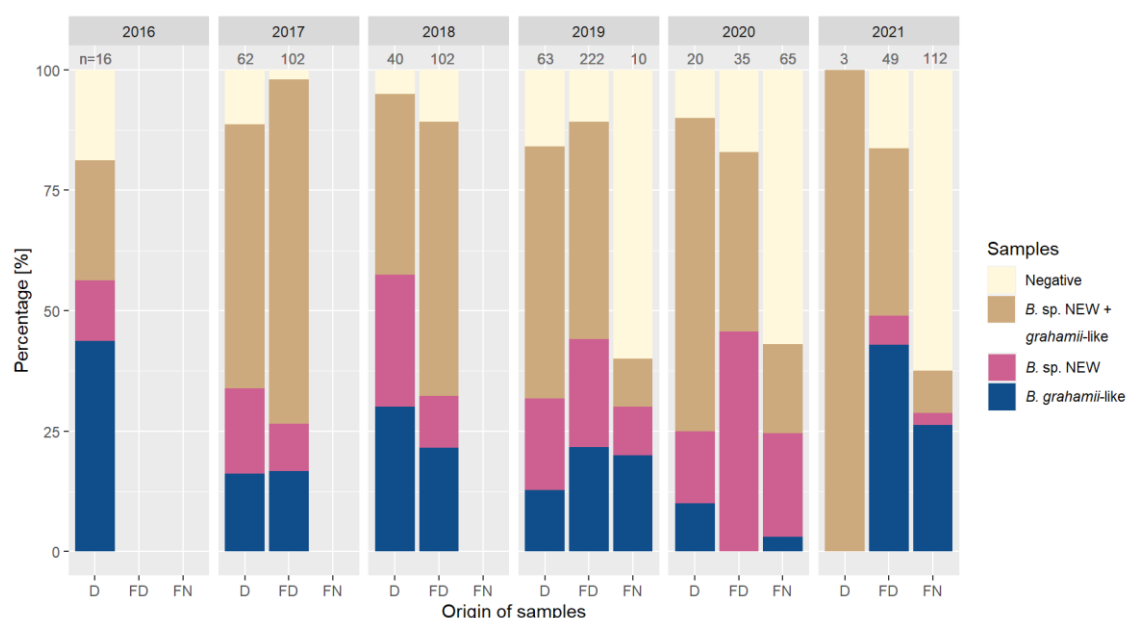


Figure 16: The fluctuation of *Bartonella* prevalence throughout the years 2016 to 2021 in samples of dormice (D) and fleas removed from dormice (FD) or nests (FN). The recognized species and mixed infections are illustrated in different colors.

The change in the occurrence of two detected species and their genotypes was monitored. Although for most of the years the ratio of the two species was balanced and fluctuated only from 40 to 60%, in some years (2016, 2021) there were more pronounced differences (Fig. 17). Altogether, the species *B. grahamii*-like was discovered to be slightly more prevalent in samples, this phenomenon is caused by the higher number of recognized genotypes of *B. grahamii*-like in short products (mixed infections are counted as all detected species). In 2016 and 2021, higher rates of *B. grahamii*-like could be influenced by a lower number of sampled and *Bartonella*-positive individuals (2016: 14 dormice, 2021: 3 dormice, 41 fleas from dormice, 30 fleas from nests). Although there was variability in particular genotypes detection over years, no specific trend or pattern was observed (Fig. 17). In addition, a high proportion of genotypes of both species was unidentified.

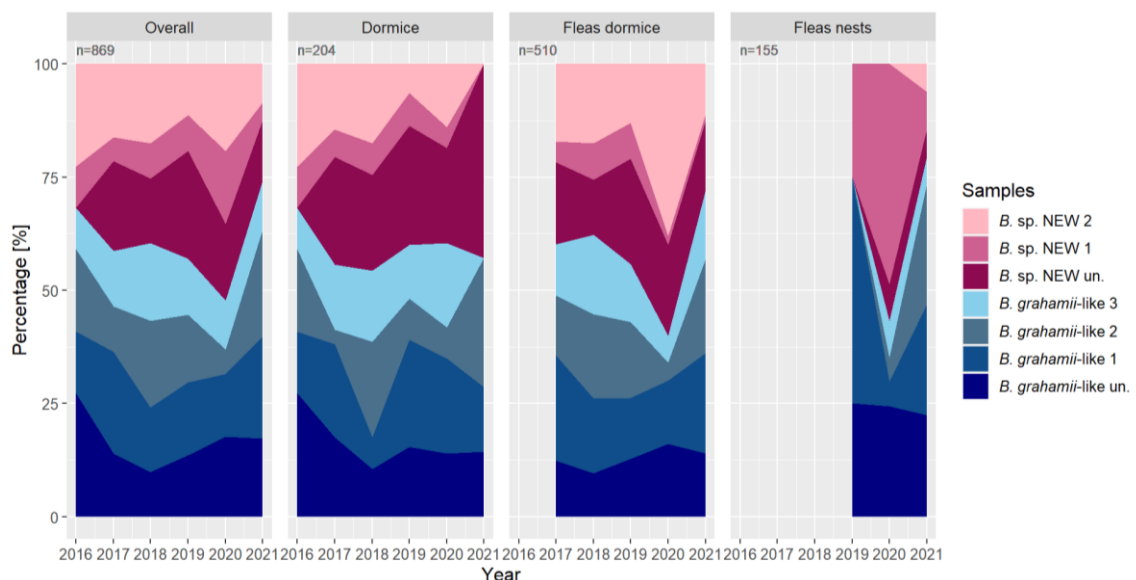


Figure 17: The prevalence of *B. grahamii*-like and *B. sp. NEW* was established by the set of PCR reactions and cultivation (genotypes identified by *gltA* PCR) in *Bartonella*-positive samples. Samples with mixed infections are counted as all the species detected in the sample and unidentified genotypes are marked as un.

### 5.2.8 Phylogenetic analyses

The long sequences of all four loci obtained from passaged clones were used in phylogenetic analyses (Fig. 18–21). The phylogenetic tree based on the concatenated sequences showed that both detected species belong to the *Bartonella* lineage 4 containing species from rodent reservoirs. Two *B. grahamii*-like genotypes clustered in close proximity to another *B. grahamii* isolate (Fig. 18). Two *B. sp. NEW* genotypes formed a sister clade to *B. washoensis* and this phylogenetic position indicates that this is a new, hitherto undescribed, species of *Bartonella*. These results are further validated by the phylogenetic tree based on the amino acid



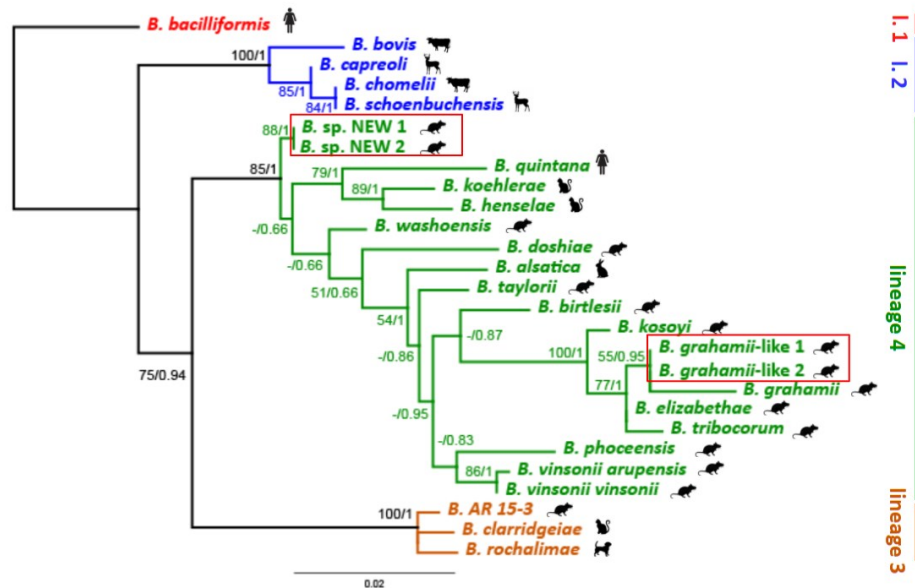


Figure 19: The phylogenetic tree of the *Bartonella* species based on the concatenated amino acid sequences of *gltA*, *rpoB*, and *ftsZ* was reconstructed using the maximum likelihood method (ML); bootstrap values for ML analysis (PhyML; 1000 replicates) and Bayesian posterior probabilities (BI, MrBayes; 5 million generations) and are shown at the nodes (dashes indicate < 50% bootstrap support or different topology). Newly obtained genotypes of *B. grahamii*-like and *B. sp. NEW* were highlighted; the tree was rooted with the *B. bacilliformis* sequence. Described lineages in bartonellae are differentiated by color; the main hosts are presented by schematic figures (representing humans, cattle, cervids, felids, canids, rodents, and leporids).

Short consensus sequences of both *gltA* and *rpoB* were used for phylogenetic analyses of only closely related species to the ones detected in our study. The phylogenetic tree based on *gltA* confirmed the position of *B. sp. NEW* as a sister clade to *B. washoensis* (Fig. 20) and also confirmed the identification of the other two detected species (both in one sample) as *B. washoensis* and ‘*C. B. rudakovii*’. However, the position of *B. grahamii*-like genotypes in the tree indicates that all three detected genotypes together with *B. sp. SCIER* (previously detected from hedgehogs and squirrels in the Czech Republic (Majerová *et al.*, 2021)) form a separate subclade.

These results were further confirmed by the phylogenetic analysis of *B. grahamii*-like sequences of *rpoB* (Fig. 21). Both *B. grahamii*-like genotypes clustered with *B. sp. SCIER* genotypes and form the new subclade sister to other species of *B. grahamii*. These results clearly indicate that *B. grahamii*-like is together with *B. sp. SCIER* a new undescribed subspecies of *B. grahamii*. This presumption is also confirmed by phylogenomic analysis performed in the Military Health Institute by Oldřich Bartoš, which showed the position of both our *B. grahamii*-like genotypes as a sister clade to all other *B. grahamii* species with whole-genome available (Fig. 22).



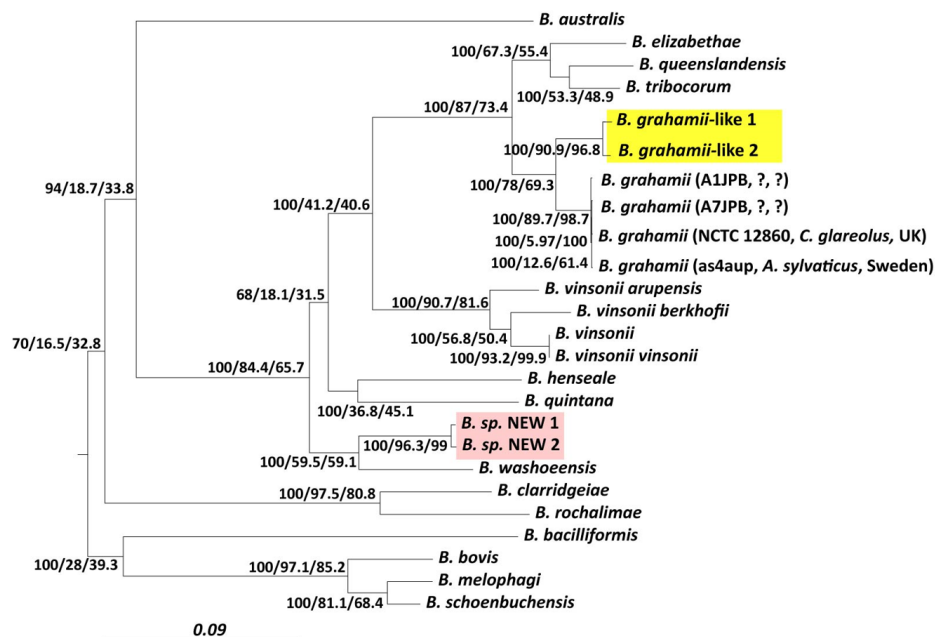


Figure 22: The phylogenetic tree based on concatenated amino acid sequences obtained from genome sequencing of *B. grahamii*-like 1, 2 and *B. sp. NEW 1*, 2 (highlighted by yellow and pink, respectively) and reference sequences (black), reconstructed using the maximum likelihood method with IQ-TREE. Genome sequencing and phylogenomic analysis were performed by Martin Chmel and Oldřich Bartoš from the Military Health Institute.

### 5.3 Bartonellae cultivation

A total of 234 samples (Tab. 18) were used for cultivation; 100, 70, and 64 samples from 2019 to 2021. Dormice blood samples, fleas sampled from dormice, and their nests were used for cultivation regularly; in 2021 also newly emerged adult fleas and flea feces samples were used. Altogether 97 (45%) samples were discarded due to contamination and no results were therefore obtained. From the rest of the samples, 69 (50%) were detected to be *Bartonella*-positive.

Table 18: The number of dormice and fleas (sampled directly from dormice, nests, and newly emerged adult fleas) used for cultivation from 2019 to 2021.

| Year    | Dormice | Fleas        |            |               |
|---------|---------|--------------|------------|---------------|
|         |         | from dormice | from nests | newly emerged |
| 2019    | 59      | 31           | 10         |               |
| 2020    | 19      | 18           | 33         |               |
| 2021    | 3       | 30           |            | 31            |
| Overall | 81      | 79           | 43         | 31            |

### 5.3.1 PCR analysis of cultivated samples

A maximum of 32 colonies per sample were analyzed. Although most colonies were analyzed by ITS-PCR (1552 colonies from 40 dormice and 12 fleas), part of the samples from summer 2019 were also analyzed by *gltA*-PCR (167 colonies from 8 dormice) and MALDI-TOF (Chapter 4.4). A total of 1719 colonies were used to identify *Bartonella* infections, not considering the first samples from the initial part of the experiment when the cultivation protocol had not yet been established and therefore was inconsistent. Prevalence of *B. grahamii*-like, *B. sp. NEW*, and mixed infections in positive samples were detected to be 35%, 38%, and 28%, respectively (Fig. 23). The different genotypes of both species were recognized in some cases using *gltA*-PCR, but later the identification was based on ITS assay only (because of time and cost-efficiency). Considering the samples with mixed infections, the colonies of both species appeared almost equally in most cases. However, sometimes only one of the analyzed colonies was detected to be of another species than all the rest.

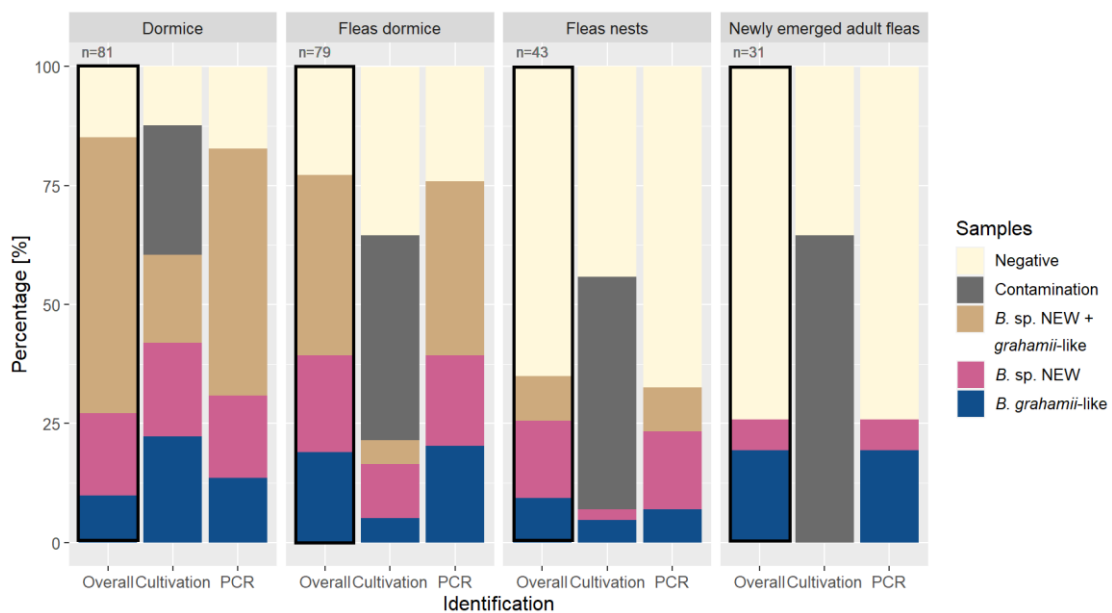


Figure 23: Comparison of results from cultivation, PCR assays of 4 loci (for newly emerged adult fleas ITS only), and combined results. Prevalence of detected *Bartonella* species, mixed infections, and contaminations (in case of cultivation) are illustrated in different colors. The samples were divided into four groups according to their origin: dormice, fleas from dormice, fleas from nests, and newly emerged adult fleas.

The evaluation by cultivation was in considerable part prevented by contamination of samples by different bacteria or fungi growing much faster than bartonellae. Altogether, 30 fleas from nests were dissected and only the digestive tract was used for cultivation. However,

using only the digestive tract did not decrease the level of contamination (57% of contamination in samples of dissected fleas, 39% in samples of whole fleas).

For a total of 203 samples, the homogenate was divided in half and used parallel for cultivation and DNA extraction for the subsequent four PCR reactions. This holds true for all cultivated samples, excluding only newly emerged adult fleas (31 samples), as they were analyzed by cultivation and ITS-PCR only. A total of 77 culture samples were contaminated, so positivity could not be assessed by cultivation, within them 55 (71%) were *Bartonella*-positive by PCR analysis. The overall *Bartonella* spp. prevalence detected by cultivation was 40%, compared to 70% revealed by PCRs. From a total of 57 samples considered negative for *Bartonella* sp. by cultivation, 23 (40%) were *Bartonella*-positive by at least one PCR protocol. On the other hand, only three samples (2 dormice blood samples, 1 sample of flea from nests) positive by cultivation were considered negative by all four PCRs.

### **5.3.2 Cultivation of newly emerged adult fleas**

Altogether, 24 samples of newly emerged adult fleas were cultivated and the growth of *Bartonella* colonies was checked. 19 samples were overgrown by bacteria contamination and therefore no assessment could be done, and the remaining five samples were *Bartonella*-negative (Fig. 23). Parallel ITS-PCR analysis confirmed *Bartonella*-positivity of eight samples. However, during cultivation, five of these samples were overgrown by contamination before *Bartonella* colonies could appear, and three were negative.

### **5.3.3 *Bartonella* clones' preparation**

Clones passaged three times were prepared from altogether 14 original samples of dormice blood, fleas from dormice, and nests; sampled in summer 2019 and 2020. From seven dormice blood samples, 14 different passaged clones were obtained and determined as *B. grahamii*-like (3 genotypes) and *B. sp. NEW* (2 genotypes). From a total of 6 original samples of fleas from dormice, 11 different clones were obtained, including both *Bartonella* species represented by two genotypes. One flea sampled from a nest was used to obtain one clone of *B. grahamii*-like 1. All obtained clones (except for the *B. grahamii*-like 3 clone) were harvested for biomass and placed at  $-70^{\circ}\text{C}$  for long-term storage.

A total of 7 samples of clones (*B. grahamii* and *B. sp. NEW*, both in two genotypes) were thawed for further analysis or viability control. All samples were able to grow under the conditions used for *Bartonella* cultivation.

## 5.4 MALDI-TOF analysis

MALDI-TOF analysis was performed for samples from summer 2019 (cultivated from autumn 2019 to summer 2020). Initially, 15 clones (*B. grahamii*-like, *B. sp. NEW*) cultivated on agar plates were used to create the *Bartonella* spp. database. Altogether, 128 colonies (from 11 different samples, using up to 16 colonies per sample) were analyzed by MALDI-TOF (Tab. 19), and the accuracy and success rate differed between the examination dates.

Table 19: The number of samples and colonies used for MALDI-TOF analysis on different dates, describing the results by the score value either below 1.7 (not accurate, invalid), ranging from 1.7 to 2 (valid identification of the genus level), or above 2 (valid identification of the species level).

|          | Original Samples | Colonies | Result score   |       |    |
|----------|------------------|----------|----------------|-------|----|
|          |                  |          | <1.7 (no data) | 1.7–2 | >2 |
| February | 4                | 64       | 3              | 9     | 52 |
| June     | 3                | 46       | 23             | 14    | 9  |
| July     | 4                | 18       | 15             | 3     | 0  |
| Overall  | 11               | 128      | 41             | 26    | 61 |

In February, a total of 64 colonies from four samples (cultured for about two months) were examined (Table 19). Only 14 of these clones were also analyzed by PCR, and all species and genotype identifications by MALDI-TOF matched the PCR results. In June, a total of 46 colonies (cultured for 6 or 4 months) originating from three samples were examined (Tab. 19). 23 colonies were not determined at all, because either the confidence score of determination was too low or no data from the samples were measured. Considering the *Bartonella* species, all colonies were determined accurately (in comparison with PCR results), for genotype determination no data were available as only the ITS-PCR assay was used. In July, altogether 18 colonies (cultured for 1 or 2 months) originating from four samples were examined, and only three measurements were successful. The rest of the colonies were not determined at all as the confidence score of determination was too low or the data from the samples were not measured.

The success rate for *Bartonella* species recognition by MALDI-TOF analysis changed from the initial 95% to 50%, and later to 17%. Not all samples were tested by PCR parallelly because the identification was found to be accurate for a score above 1.7. In all samples analyzed by both assays (PCR and MALDI-TOF), the species determination was identical.

## 5.5 Characterization of *Bartonella* species

Two species, *B. grahamii*-like and *B. sp. NEW* were cultivated and both were detected in several genotypes (*B. grahamii*-like 1–3; *B. sp. NEW* 1, 2). However, the genotype *B. grahamii*-like 3 was obtained later in the study and was not, therefore, used for species characterization.

### 5.5.1 Colony morphology

The colonies size of both *Bartonella* species was 0.6 to 3 mm depending (partially) on the number of passages. However, morphological heterogeneity was observed for both species (Fig. 24 A, B). The colonies that were frozen and thawed usually grew to smaller sizes and a larger number of colonies formed a thin layer on the agar plate. The consistency of the colonies varied from hard and attached to the surface to creamy ones that were easily removed. The colonies were circular with sharp edges and the color was mostly white-yellowish (Fig. 24).

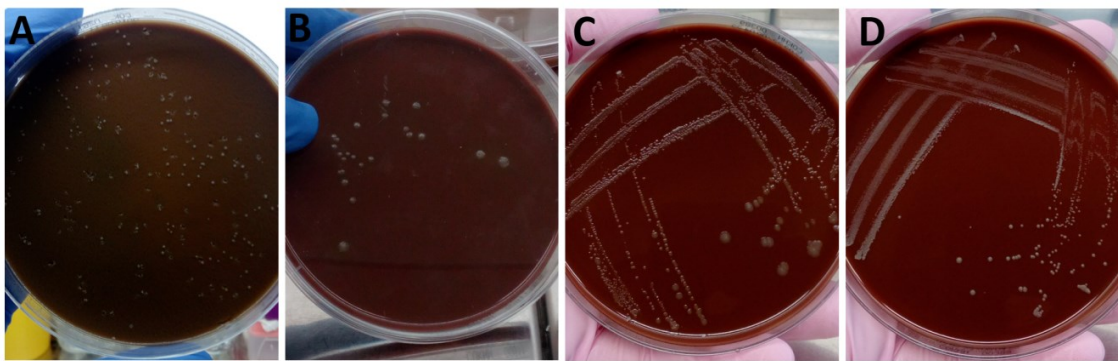


Figure 24: Pictures of the cultivated colonies in different sizes, morphotypes, and passages. A, B: original sample (0 passages), C, D: thawed passaged clones (several passages after thawing).

### 5.5.2 Characterization of bacteria by TEM

Transmission electron microscopy (TEM) was performed for both species *B. grahamii*-like and *B. sp. NEW* (both in two genotypes), and the results showed rod-shaped bacilli without any pili or flagella. The size of the bacteria did not differ between species and ranged in length from 0.8 to 1.3  $\mu\text{m}$  (mean = 1.1  $\mu\text{m}$ , SD = 0.17  $\mu\text{m}$ ) and the width from 0.3 to 0.5  $\mu\text{m}$  (mean = 0.36  $\mu\text{m}$ , SD = 0.06  $\mu\text{m}$ ).

#### 5.5.2.1 Negative staining

The staining of *B. grahamii*-like and *B. sp. NEW* (both in two genotypes) was successful, and the results were examined under TEM. The bacteria measurements were taken, and the

morphological differences were examined (Fig. 25). Morphological examination showed that neither the two species nor their genotypes have any observable morphological differences.

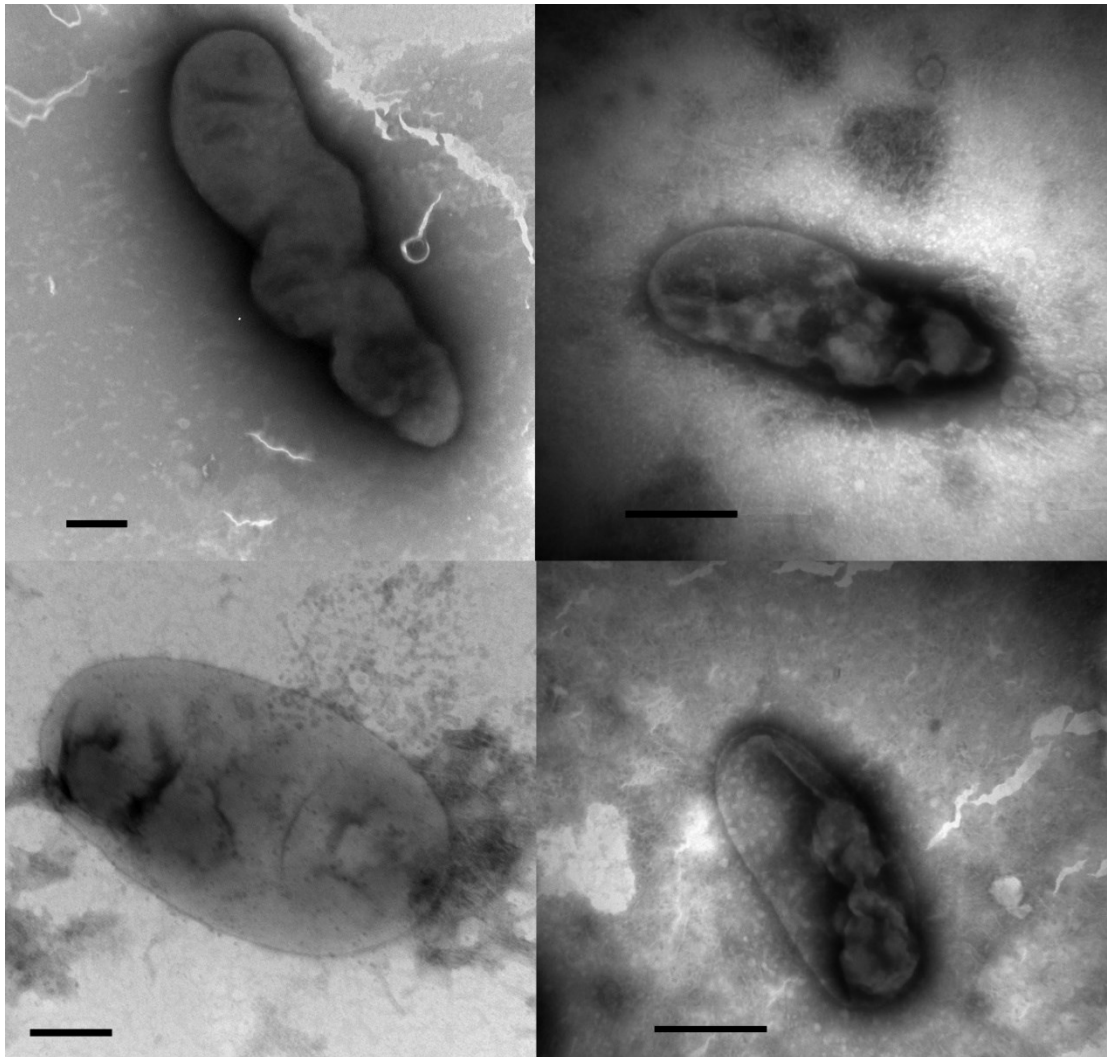


Figure 25: The sample of A: *B. sp. NEW 1* (scale bar: 400nm), B: *B. sp. NEW 2* (scale bar: 400nm), C: *B. grahamii*-like 1 (scale bar: 200nm), and D: *B. grahamii*-like 2; *Bartonella sp.* imprint (scale bar: 400nm), prepared by negative staining by phosphotungstic acid, visualized by TEM (JEM 200CX Jeol).

#### 5.5.2.2 Fixation and embedding

Both genotypes of the species *B. grahamii*-like and *B. sp. NEW* were prepared by fixation and embedding. However, not all samples were prepared successfully because a low amount of biomass was obtained from agar plates (due to the slow-growing nature of bartonellae) and a substantial volume was lost due to processing, therefore embedding was not possible. Only samples of *B. grahamii*-like 1 and *B. sp. NEW 2* were successfully embedded and visualized under the TEM (Fig. 26). The morphology of both species was examined, and no obvious differences were observed.

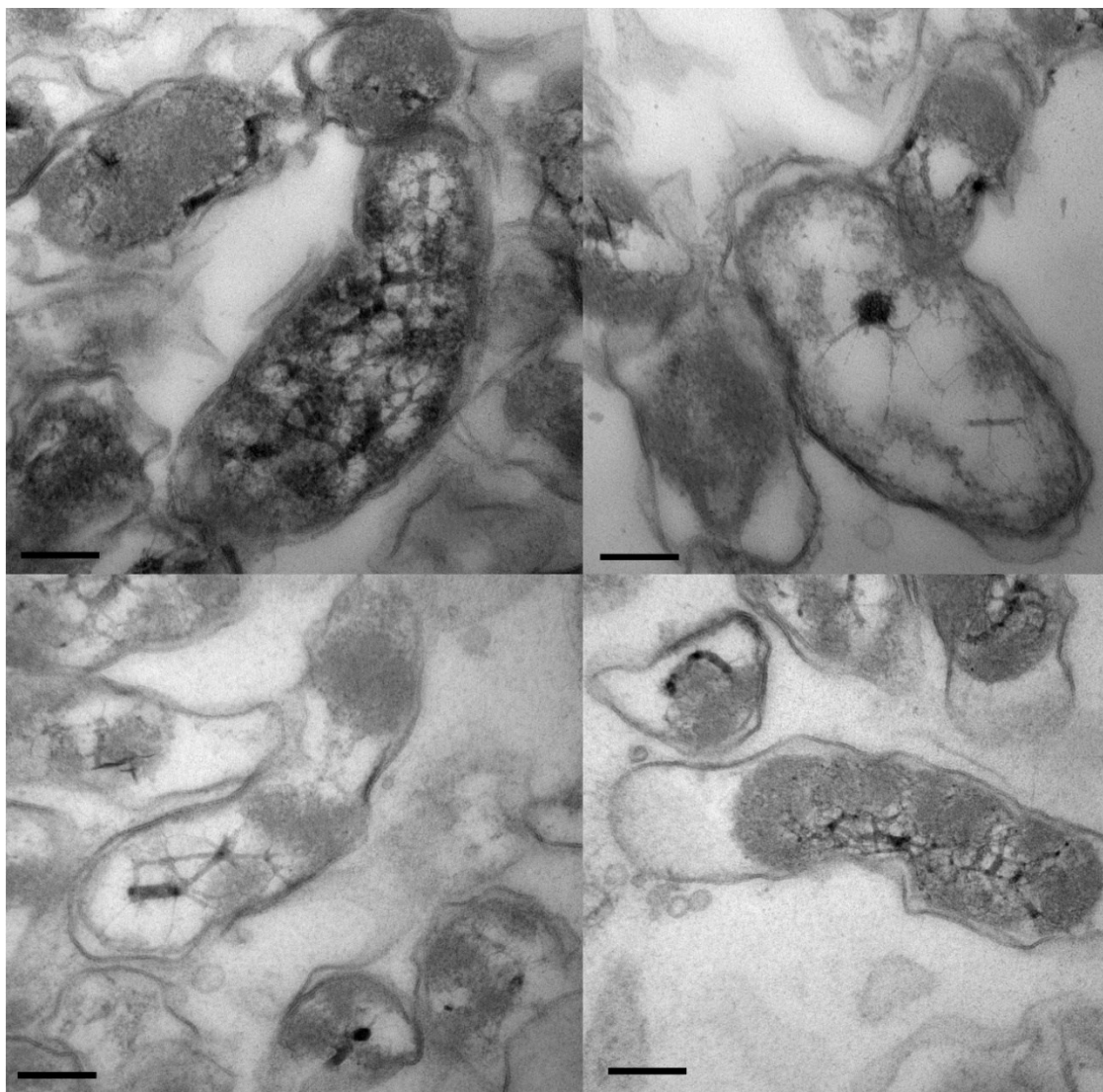


Figure 26: The samples of *B. grahamii*-like 1 (A, B) and *B. sp. NEW 2* (C, D) prepared by fixation and embedding and visualized (Scale bar: 200 nm) under TEM (JEM 200CX Jeol).

### 5.5.3 Temperature and CO<sub>2</sub> requirements

Two genotypes of each *B. grahamii*-like and *B. sp. NEW* were inoculated to new agar plates (6 plates per each genotype) and cultured under different conditions. Although the growth rate differed between species in several cases, the differences were rather caused by the variation in inoculation than by the difference in the growth rate between species/genotypes.

The results showed the ability of passaged *Bartonella* of both species to grow to fully grown colonies in 8 to 10 days at 37 °C with 5% CO<sub>2</sub> (standard conditions used in the cultivation protocol). The growth rate was observed to be steady and on the second day, small colonies were already present on the plates (Fig. 27, 28). Although at 32 °C with 5% CO<sub>2</sub> colonies were also fully grown in 10 days, compared to plates at 37 °C (5% CO<sub>2</sub>), half of the plates contained

a significantly lower amount of biomass (Fig. 28). The growth rate was slower and on the second day, the colonies were not observable on any plate. At 42 °C with 5% CO<sub>2</sub> colonies were observed only on two plates on day 8 and only a few colonies were able to grow. Altogether, 8 plates were cultivated at 37 °C without any added CO<sub>2</sub>, and only on three plates, some colonies were observed: two plates had just a few colonies after 10 days of growth; however, the third plate showed growth similar to that at 32 °C with added CO<sub>2</sub>.

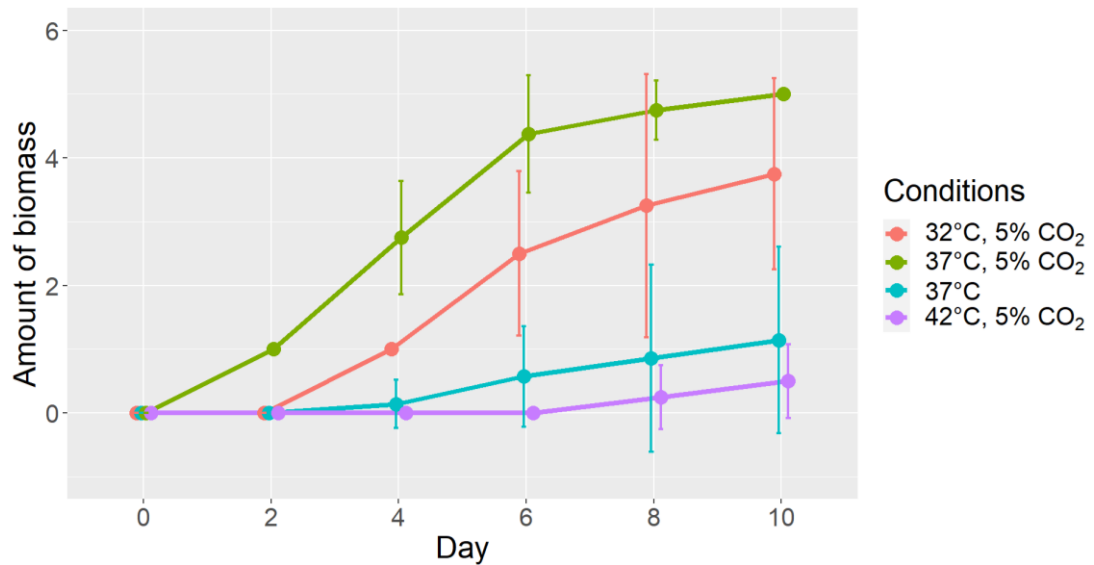


Figure 27: The average biomass coverage (scale 1–5; see Fig. 28) for plates grown under different conditions and evaluated over ten days of cultivation.

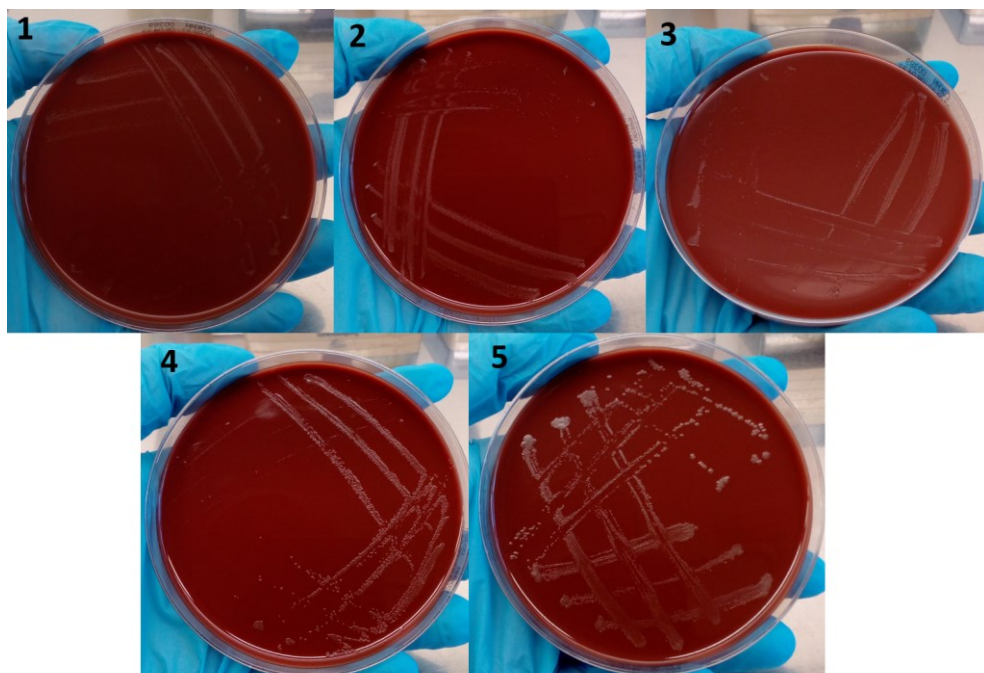


Figure 28: Illustration of the evaluation scale (from 1 to 5) for growth experiment under different conditions of bartonellae biomass on agar plates.

## 6 Discussion

### 6.1 Prevalence and diversity of *Bartonella* spp. in dormice and fleas

To date, only one study (unpublished diploma thesis) focused on bartonellae of edible dormice and their fleas (Bundilová, 2020). Our project follows up on the results of this work (e. g. samples from the years 2016–2018 were used for both studies) and provides detailed insights into *Bartonella* spp. prevalence and diversity, as well as a comparison of various detection and identification methods.

Although the overall *Bartonella* spp. prevalence determined in our study for dormice and fleas collected from dormice were very similar (89% and 90%, respectively), but the prevalence detected in fleas collected from dormice nests was lower (40%). In addition, a lower rate of mixed infection was also found in fleas from nests. The observed differences suggest that most of the fleas collected from dormice nests have not yet taken any blood meals and did not obtain the bartonellae infection via transstadial transmission, as flea infection is described as lifelong (Bouhsira *et al.*, 2013; Morick *et al.*, 2013a).

#### 6.1.1 Diversity of detected bartonellae

Four different bartonellae species (two most abundant in several genotypes) were identified in dormice and their fleas, all belonging to lineage 4, typical for rodent species. *Bartonella* sp. NEW was identified as a novel species according to the threshold criteria proposed by La Scola *et al.*, 2003 (identity in sequences of partial gene *gltA* < 96% and *rpoB* < 95.4%): the closest GenBank match, *B. washoensis* has 92% similarity in *gltA* and 94% similarity in *rpoB* partial gene sequences proposed to be discriminatory. According to the phylogenetic analyses, the common *B. sp. NEW* and the rare *B. washoensis* (found in one flea only) form a separate clade. *B. washoensis* is associated mainly with the family Sciuridae (Stevenson *et al.*, 2003; Inoue *et al.*, 2011; von Loewenich *et al.*, 2019; Lipatova *et al.*, 2020; Majerová *et al.*, 2021). However, *B. washoensis* detection in hedgehogs (*Erinaceus europaeus*, Czech Republic) and dogs (USA) questions the host specificity (Chomel *et al.*, 2003; Majerová *et al.*, 2021). In addition, *B. washoensis* was also detected in humans (USA and Germany) causing clinical symptoms (fever and myocarditis) (Kosoy *et al.*, 2003; Probert *et al.*, 2009; von Loewenich *et al.*, 2019).

While four genotypes of *B. sp. NEW* represent a completely new species, *B. grahamii*-like genotypes should be considered as a new subspecies within the *B. grahamii* clade. This presumption is supported by the phylogenetic position and distances of three described subspecies of *B. vinsonii* (*vinsonii*, *arupensis*, and *berkhofii*) which is comparable to the

distance between *B. grahamii* and *B. grahamii*-like. On the other hand, *B. grahamii*-like genotypes showed the similarity of 98.3–99% in *gltA* and 96.5–96.7% in *rpoB* partial gene sequences mentioned by La Scola *et al.* (2003) to reference *B. grahamii* sequences in the GenBank database. In comparison, the similarity of *B. vinsonii* subspecies varies for *gltA* from 96.7 to 96.9% and for *rpoB* from 94.5 to 96.2%, showing more differences among the *B. vinsonii* complex than the one described in our study within the clade *B. grahamii*. Previous studies described a high polymorphism of *B. grahamii* with a strong geographical pattern, describing higher diversity in Asia compared to Europe (Berglund *et al.*, 2010b; Mardosaitė-Busaitienė *et al.*, 2019b). Only four *B. grahamii* genotypes have been described in different rodent species (e. g. *Apodemus agrarius*, *Clethrionomys glareolus*) at various localities in Lithuania (Mardosaitė-Busaitienė *et al.*, 2019a). However, our study described the same diversity (4 genotypes) of *B. grahamii*-like from one locality in only one rodent species, indicating higher diversity of *B. grahamii*, than was expected. The higher diversity of *B. grahamii*-like described in our study could be influenced by the detailed detection methods used for *Bartonella* spp. detection.

*B. grahamii* was detected in various rodent species almost all over the world (Asia, Europe, and America) and is considered to be one of *Bartonella* species with the highest prevalence in rodents (Bown *et al.*, 2004; Inoue *et al.*, 2009; Berglund *et al.*, 2010b; Mardosaitė-Busaitienė *et al.*, 2019a; Obiegala *et al.*, 2021; Rao *et al.*, 2021). Moreover, *B. grahamii* has been described to cause symptomatic infections similar to the cat-scratch disease as well as neuroretinitis, particularly in immunosuppressed patients (Kerkhoff *et al.*, 1999; Drancourt *et al.*, 2008; Terrada *et al.*, 2009; Oksi *et al.*, 2013). The phylogenetic analyses of short sequences of *rpoB* and *gltA* loci showed that our genotypes of *B. grahamii*-like are closely related (and in some cases identical) to *Bartonella* sp. SCIER genotypes, previously detected in squirrels and hedgehogs in the Czech Republic (Majerová *et al.*, 2021).

As dormice and hedgehogs do not share the same species of ectoparasites (for hedgehogs are typical *Ixodes hexagonus* ticks and *Archeopsylla erinacei* fleas), are not closely related, and do not carry the same spectrum of *Bartonella* spp. (except for *B. washoensis*) (Földvári *et al.*, 2011; Szekeres *et al.*, 2019), the detection of the same/similar species (*B. sp.* SCIER / *B. grahamii*-like) (Majerová *et al.*, 2021; this study) was unexpected and the association between dormice and squirrels on one side and hedgehogs on the other side remains unclear. However, the recognized host spectra for most of the *Bartonella* species are continuously growing and, in some cases, comprise ecologically and taxonomically unrelated host species (Breitschwerdt, 2017; Cheslock & Embers, 2019).

The fourth species detected in our study, '*C. B. rudakovii*', was previously found in several other rodent species: *C. glareolus*, *Microtus oeconomus*, *A. agrarius* (Lithuania, Switzerland), and *Sciurus vulgaris* (Czech Republic) (Mardosaitė-Busaitienė *et al.*, 2019a; Divari *et al.*, 2021; Majerová *et al.*, 2021). The detection of '*C. B. rudakovii*' in dormice corresponds to the so far described rodent-restricted host spectrum and extends it for additional species.

A similar diversity of *Bartonella* species (*B. washoensis*, '*C. B. rudakovii*', *B. grahamii*, and *B. sp. SCIER* (= *B. grahamii*-like)) was detected in squirrels in the Czech Republic, UK, USA, and Lithuania (Bown *et al.*, 2002; Inoue *et al.*, 2011; Lipatova *et al.*, 2020; Majerová *et al.*, 2021). Another study described additional species from *S. vulgaris* and *S. carolinensis* (UK, USA), one species was identified as *B. vinsonii* clade, and for the other, the phylogenetic classification was unclear (Bown *et al.*, 2002). Because the *Bartonella* species detected in our dormice and their fleas showed an association with squirrels, it is likely that the *Bartonella* species exchange between these hosts. Although dormice and squirrels are not closely related (however, they belong to the same suborder Sciuromorpha), both share a similar environment and also the same flea species, *Ceratophyllus sciurorum* (discussed below) (Barthelmess *et al.*, 2016).

In dormice, Bundilová showed the presence of two species (without recognizing genotypes) of *Bartonella* (*B. grahamii* and *Bartonella* sp., which are referred to as *B. grahamii*-like and *B. sp. NEW* in our study). However, Bundilová's study did not focus on the phylogenetic analysis of detected species and therefore did not describe their phylogenetic relationships. In our study, seven genotypes (of these two species) were detected in dormice blood. Similar diversity was found in *Onychomys leucogaster* populations (USA), with a detected 90% prevalence of bartonellae carrying infections of ten *Bartonella* genotypes, clustering into four phylogenetic variants (most likely species) (Bai *et al.*, 2007). However, their research was conducted on four geographically different localities, which could increase the described diversity. In our study, the sampling took place at one locality only; therefore, the dormice of one population were sampled.

Although seven genotypes (belonging to two species) were detected in dormice blood, 11 genotypes of four species were found in fleas. However, the results are influenced by the much higher number of sampled fleas (823) compared to dormice (203). As all the additional genotypes (and two species) were detected only in one flea each, the difference in diversity (dormice vs. fleas) could be caused by chance. A higher diversity of *Bartonella* genotypes in fleas compared to the hosts was described also in other studies, detecting six abundant genotypes in fleas and only two in rodent hosts *Gerbillus andersoni* (Israel) (Gutiérrez *et al.*, 2014b). These results suggest that mammals are exposed by fleas to a broad spectrum of

*Bartonella* spp., which does not result in bacteremia in some cases (Abbot *et al.*, 2007). The diversity of *Bartonella* species in rodent infections is influenced by the host specificity of their ectoparasites, which can transmit bacteria between vertebrate host species. *Ceratophyllus sciurorum* was the dominant flea species found on dormice in our study. Although the species specificity of this flea is described as low (small mammals, birds, humans) (Rosický, 1957); its close association with dormice and squirrels (e. g. *Sciurus vulgaris*) (Cyprich *et al.*, 1992; Lipatova *et al.*, 2020) further indicates the possible exchange of *Bartonella* spp. between squirrels and dormice. In addition, ticks were described to be abundant on dormice (Matuschka *et al.*, 1994) and previous studies confirmed the role of ticks as competent vectors of some *Bartonella* spp. (Billeter *et al.*, 2008; Cotté *et al.*, 2008; Reis *et al.*, 2011). However, the ticks were not sampled in our study, and their potential role in bartonellae transmission was not examined. Ticks as vectors could potentially explain the occurrence of some *Bartonella* species (e. g. *B. washoensis* and *B. grahamii*-like) in dormice and hedgehogs or dogs.

Unlike the phylogenetic analysis based on three concatenated amino acids sequences (*gltA*, *rpoB*, and *ftsZ*), the amino acid phylogenomic analysis (provided by O. Bartoš) recognized obvious heterogeneity between the genotypes of both species tested. As the cultivation of *B. grahamii*-like and *B. sp. NEW* was successful (three and two genotypes) after additional biochemical characterization, all required information will be available for the formal description of new (sub)species.

### **6.1.2 Methods for the detection of bartonellae**

The prevalence of *Bartonella* spp. detected in various rodent species was described to reach up to 90% and for fleas associated with rodents up to 70%, depending not only on the rodent/vector ecology but also on the methods used for the detection. Various methods are commonly used in bartonellae screening studies of free-living animals, ranging from conventional and real-time PCR targeting a various number of loci to cultivation (Bai *et al.*, 2007; Morway *et al.*, 2008; Gutiérrez *et al.*, 2018; Mardosaitė-Busaitienė *et al.*, 2019a).

The prevalence detected in Bundilová's work was 53% for dormice and 44% for fleas associated with dormice, compared to the prevalence reaching 90% for both dormice and fleas established in our study. The previous significantly lower prevalence detected in the same samples (in part) was caused by different protocols for *Bartonella* spp. detection – analysis using one-step PCR targeting *gltA* and ITS (with different primers) (Bundilová, 2020). Although Bundilová's samples were evaluated by PCR targeting the *gltA* and ITS loci that were shown in our study to detect the highest prevalence of *Bartonella*, the use of one-step PCR prevented the recognition of infections in almost half of the samples.

Similar results were described in studies detecting bartonellae in *A. agrarius* (Russia, Slovakia) using one-step or nested PCR describing the prevalence of 9–27% and 73%, respectively (Mediannikov *et al.*, 2005; Kraljik *et al.*, 2016). On the other hand, a high *Bartonella* spp. prevalence (63%) in *A. flavicollis* (Slovenia) was described even with the use of one-step PCR (Knap *et al.*, 2007). In addition, the *Bartonella* spp. prevalence of 90% in *O. leucogaster* (USA) was detected by cultivation and one-step PCR targeting *gltA* (Bai *et al.*, 2007). Although the protocols used for *Bartonella* spp. detection were less complex than those used in our study on dormice, the detected prevalence reached the same rate. This could be caused by higher bacteremia in *A. flavicollis* that was possible to detect even with one-step PCR and a more successful cultivation technique of *O. leucogaster* samples probably due to different blood sampling techniques (blood from the retroorbital plexus).

In our study on dormice, the detection of *Bartonella*-positive samples by cultivation described the prevalence of 40% from the samples of both dormice and fleas compared to the 70% prevalence detected by PCR in the samples used for cultivation. Similar results were obtained from cat blood samples (Israel) when a real-time PCR assay detected 94% positive samples and cultivation only 36% (Gutiérrez *et al.*, 2013). However, through cultivation, only the presence of viable *Bartonella* spp. is determined, and therefore the difference between cultivation and PCR methods could be caused by the DNA detection of bartonellae that are no longer able to grow. Furthermore, bartonellae ability to grow and multiply could decrease due to the storage of samples before cultivation (blood samples at –20 °C).

In our study, 27% and 49% of all dormice and flea cultivated samples, respectively, were discarded due to contamination and therefore no relevant results were obtained. Although problems with contaminations were described in some studies on rodents, the rate of discarded samples due to contamination was much lower, approximately 12%. However, blood was taken from dead or freshly killed animals, allowing more sterile blood sampling (Kosoy, 1997).

In our study, *Bartonella* spp. detection was performed on blood samples taken from live animals in which the bacteremia levels can vary in time due to the relapsing nature of bartonellae infections (Okujava *et al.*, 2014). The use of internal organs (e. g. lung, bladder, and mostly spleen) for *Bartonella* spp. detection was also tested in the case of animal cadavers, showing a high efficiency (Kraljik *et al.*, 2016; Lipatova *et al.*, 2020; Majerová *et al.*, 2021). However, bartonellae are blood parasites with endothelial and erythrocytes tropism and therefore no organ specificity is expected (Okujava *et al.*, 2014; Majerová *et al.*, 2021).

Our PCR analysis targeting four loci (*gltA*, *rpoB*, *ftsZ*, and ITS) was used for the recognition and description of bartonellae infection. Although the loci *gltA* and ITS showed the

best overall results (polymorphism, prevalence detection, specificity to detected species, cost-effectiveness), each of the chosen PCR protocols together with cultivation revealed positivity or mixed infection in additional samples, which would be missed by all other protocols.

The recognition rate of mixed infection for single loci ranged from 1 to 23%. With the combination of all the methods used, the mixed infection rate was 44% indicating a high tolerance of dormice and fleas to harbor mixed infections. A similar mixed infection rate (46%) was described in *Gerbillus* spp. (Israel) using PCR analysis targeting loci *gltA*, *rpoB*, and ITS in combination with cultivation (Gutiérrez *et al.*, 2018). Even a higher rate of mixed infections (89%) was described by *gltA*-PCR in *G. andersoni* (Israel) (Gutiérrez *et al.*, 2014b). It is discussed that detailed analysis targeting several loci and/or cultivation is needed for the detection of mixed infections (Gutiérrez *et al.*, 2018).

### **6.1.3 The effect of host and vector ecology on the prevalence of bartonellae**

In Germany, edible dormice were described to act as good reservoir hosts for *Borrelia burgdorferi* mainly due to their longevity (up to 9 years), high level of infestations with ectoparasites, and abundance (Matuschka *et al.*, 1994). These factors could also contribute to the high prevalence of *Bartonella* spp. described in our study and the competence of edible dormice as hosts.

In comparison, the prevalence of bartonellae in North American Richardson's ground squirrel (*Urocitellus richardsonii*) with a long-life span (females up to 6 years) (Michener, 1998) was detected to be 74% over several years (Jardine *et al.*, 2006a). *Bartonella* spp. prevalence of 90% and 80% were described in *O. leucogaster* (USA) and *A. flavicolis* (Lithuania), respectively (Bai *et al.*, 2007; Mardosaitė-Busaitienė *et al.*, 2019a). The high detected prevalence was attributed to species-specific behavior in the case of *O. leucogaster*, such as predatory behavior and taking over other small mammals' burrows, leading to a higher probability of bartonellae acquisition (Bai *et al.*, 2007). In the case of *A. flavicolis*, the high detected prevalence was attributed to various habitats inhabited by the examined population of rodents (Mardosaitė-Busaitienė *et al.*, 2019a). Although dormice predate birds and take over their nests (Adamík & Král, 2008b), so far, no *Bartonella* species have been described in birds.

Infestation of dormice with fleas was described as rather high (Lipatova *et al.*, 2015) and this observation was confirmed in our study. Although some studies attribute a higher *Bartonella* spp. prevalence to a higher infestation rate with rodent ectoparasites (Welc-Faleciak *et al.*, 2010), other studies did not find any association between *Bartonella* spp. prevalence and flea loads (Jardine *et al.*, 2006b; Morway *et al.*, 2008). The lack of relationship

is discussed because of two possible routes of *Bartonella* spp. transmission from fleas to rodents (by feces and during blood feeding), the high amount of bartonellae needed to induce infection in hosts, and the different methods used to detect bartonellae in rodents and fleas (Morway *et al.*, 2008). A study tested the effect of infestation rates on the different *Bartonella* spp. prevalence in *Rattus rattus* and *R. norvegicus* (South Africa). The results showed that only a small part (3 to 5%) of the overall difference (19%) in prevalence can be attributed to different levels of flea infestation (Brettschneider *et al.*, 2012). Therefore, it could be expected that the high *Bartonella* spp. prevalence detected in dormice in our study is at least partially attributed to the high infestation rates.

The relationships between the bartonellae infections detected in dormice and their fleas were not analyzed, because fleas change their host often in the nest and therefore no long-term association is expected. The detected 90% prevalence of *Bartonella* spp. in fleas corresponds to the detected prevalence in dormice (89%). A similar trend was described for *G. andersoni* (Israel), the prevalence was detected for rodents and fleas at 75 and 71%, respectively (Gutiérrez *et al.*, 2014b). In Lithuania, *Bartonella* spp. prevalence of 56% was detected in fleas *C. sciurorum*; however, the prevalence detected in squirrels was lower (39%) (Lipatova *et al.*, 2020).

Although the abundance of dormice was not examined in our study, the effect of host density on pathogen prevalence was examined in previous studies. A detailed analysis of *Bartonella* spp. prevalence and rodent host abundance (*C. glareolus* and *A. sylvaticus*, UK) revealed direct and delayed density dependence, depending on *Bartonella* species; the delayed density dependence is due to the intermediate effect of vector abundance (Telfer *et al.*, 2007).

Our study on dormice was conducted over six years; however, only small fluctuations in the overall prevalence detected in both dormice and their fleas were observed. No apparent trend was observed in the change of relative proportion of the detected species (*B. grahamii*-like and *B. sp. NEW*) and/or their genotypes. The lack of repeated dormice samplings (retrap) can be explained by the fact that only a part of the dormice population in the locality uses the nest boxes as a shelter. The majority of dormice use cavities in trees as nests or rest sites (Juškaitis, 2005; Fedyń *et al.*, 2021). In a study on *C. ludovicianus* (USA), the annual change in *Bartonella* spp. prevalence was detected and discussed as being caused by a plague epidemic that killed many older animals and mostly naïve juveniles survived (Bai *et al.*, 2008). The small fluctuations in *Bartonella* spp. prevalence detected in our study could be caused by a small number of individuals sampled in some years (2016: 16, 2021: 3) and/or the lack of significant

change in the structure of the dormice or vector populations. To describe possible prevalence fluctuations in the dormice population, more individuals should be sampled.

#### **6.1.3.1 The effect of rodent and flea sex and age on the prevalence of bartonellae**

The prevalence of bartonellae in rodents has been described to vary between age groups. A higher prevalence in juveniles compared to adults was observed for *C. glareolus* and *M. oeconomus* (Poland), attributed to the clearance of infections and resistance, or the death of infected adults, as well as the immune naivety of juveniles (Fichet-Calvet *et al.*, 2000; Jardine *et al.*, 2006b; Welc-Faleciak *et al.*, 2010). Our finding of a significant difference between the prevalence of juveniles (79%) and adults (94%) indicates either a high adaptation of dormice to *Bartonella* spp. infection, the absence of acquired resistance, and/or clearance of the infection in older animals. The lower prevalence of *Bartonella* spp. in juveniles could be caused by the fewer opportunities to acquire and accumulate bartonellae infections, as a certain load of bacteria must be inoculated for detectable bacteremia to develop (Morway *et al.*, 2008). Furthermore, the possibility of congenital transmission between rodents was described in the case of *Sigmodon hispidus* and *Peromyscus leucopus* (USA) (Kosoy *et al.*, 1998), and the lower prevalence of juvenile dormice detected in our study could be caused by the lack of this transmission route. A similar pattern was described in another study, where the youngest individuals (according to weight, under 300 g) of *C. ludovicianus* (USA) showed a lower prevalence of *Bartonella* spp. that increased rapidly, with the highest prevalence detected at weight 300 to 400 g (Bai *et al.*, 2008). A similar trend could be expected in dormice, although a decrease in *Bartonella* spp. prevalence was not determined at a higher age as only two age categories were distinguished: juveniles and adults. Therefore, a more detailed evaluation of dormice age and repeated samplings would be necessary for the description of the *Bartonella* spp. prevalence-dormice age relationship.

In general, no significant difference was observed between the prevalence of bartonellae in dormice females and males. Several studies described a similar situation for other rodent species (Fichet-Calvet *et al.*, 2000; Kosoy *et al.*, 2004; Morway *et al.*, 2008). Although a study on *A. flavicollis* (Poland) described a higher *Bartonella* spp. prevalence in males, the reasons are unclear (Welc-Faleciak *et al.*, 2010).

In our study, *Bartonella* spp. prevalence did not differ between sexes of fleas; however, Bundilová (2020) showed a higher prevalence of *Bartonella* spp. in female fleas. Bundilová used only one-step PCR for *Bartonella* detection and therefore bartonellae infection was missed in almost half of the samples (compared to our results, discussed above). In general, the higher prevalence of *Bartonella* spp. or any other blood parasites in flea females

could be caused by their higher blood intake (higher energy requirements) connected with a higher probability of infection (Krasnov *et al.*, 2008). Therefore, it can be argued that the higher *Bartonella* spp. prevalence detected in female fleas by Bundilová could be caused by higher bacteremia in females due to higher blood intake. On the other hand, another study on ectoparasites (fleas, ticks) of dogs and cats (Taiwan) did not find any difference in *Bartonella* spp. prevalence between females and males of neither fleas nor ticks (Tsai *et al.*, 2011b).

## 6.2 Bartonellae infection in different life stages of fleas

To elucidate possible transmission routes of *Bartonella* spp. between different flea life stages, primary adults inhabited dormice nests, larvae, and newly emerged adult fleas, as well as adult flea feces were examined for the bartonellae presence.

Previous studies confirmed the presence and viability of at least some *Bartonella* spp. in flea feces and it represents one of the transmission routes to vertebrate hosts (Raoult & Roux, 1999; Bouhsira *et al.*, 2013). In our study, the viability of *Bartonella* spp. in flea feces were tested by cultivation. Especially due to the increased mortality caused (most probably) by the washing of fleas prior to defecation, obtaining flea feces was a challenge. However, all cultures were overgrown by contamination that prevented the examination of the presence of *Bartonella* spp.

Flea larvae were tested for *Bartonella* spp. presence to determine the possible non-transovarial vertical transmission of *Bartonella* spp. between fleas via infected feces, and 30% of larvae were found to be positive for bartonellae infection. Previous studies confirmed vertical transmission via infected feces of adult fleas on which the larvae fed (Morick *et al.*, 2013a). On the other hand, the possibility of transovarial transmission in our study cannot be dismissed, although this transmission route of *Bartonella* spp. in fleas has not been described so far and contradictory results are available: *Bartonella* spp. was detected in reproductive tissue of several flea species (e. g. *Oropsylla hirsuta*); on the other hand, eggs and hatched larvae from eggs laid by infected flea females (*Xenopsylla ramesis*) were detected to be *Bartonella* negative (Brinkerhoff *et al.*, 2010; Morick *et al.*, 2011). The contradictory results could be explained by hypothetical species-specificity in the transovarial transmission of *Bartonella* spp. in fleas (Gutiérrez *et al.*, 2015).

The transstadial transmission of *Bartonella* spp. in fleas was tested by examination of newly emerged adult fleas for bacteria presence. Overall, 19% of newly emerging adult fleas tested positive for bartonellae infection, confirming transstadial transmission from infected

larvae to adult fleas. However, since the flea hatching environment was in nests, sampling errors may have occurred (such as overlooked primary adult fleas sampled as newly emerged adult fleas). On the other hand, precautions were taken to minimize the risk of sampling errors, and the nests were repeatedly checked for flea presence. Even if a few primary adult fleas would be overlooked and counted as newly emerged fleas, the number of detected *Bartonella*-positive newly emerged adult fleas (22 individuals) was high and therefore the results would be still reliable. Furthermore, since the cultivation of *Bartonella* spp. from newly emerged adult fleas was unsuccessful, it is not clear whether the bacteria detected by PCR are viable. Transstadial transmission of bartonellae was previously confirmed in *Xenopsylla ramesis*, via detection of *Bartonella* spp. in newly emerged adult fleas from larvae that fed on infected feces and gut voids (regurgitation of indigested blood meals). As only PCR was used for *Bartonella* spp. detection, the viability of detected bacteria remains unknown (Morick *et al.*, 2013a).

The prevalence of *Bartonella* spp. in primary adult fleas, larvae, and newly emerged adult fleas was described to be 31%, 30%, and 17%, respectively. No significant differences in the prevalence of the examined life stages of fleas were found. As only a relatively small number of nests were used for analysis with a low number of fleas (in some cases less than 10) a high variability of prevalence between nests and flea stages was noted. More observations are needed for a thorough analysis. However, the rate of *Bartonella*-positive newly emerged adult fleas indicates that transstadial transmission is an effective transmission route of *Bartonella* spp. Therefore, it would be expected that *Bartonella* spp. could circulate in flea populations long-term without the need for vertebrate hosts to maintain bartonellae infections.

## 6.3 Cultivation and characterization of *Bartonella* clones

*Bartonella* clones passaged three times (*B. sp.* NEW and *B. grahamii*-like, both represented by two genotypes) were obtained from cultivated samples.

### 6.3.1 MALDI-TOF

MALDI-TOF is not yet a widely used technique for bartonellae detection in screening studies of free-living animals (Fournier *et al.*, 2009). Although most of the cultured samples were identified by ITS-PCR, MALDI-TOF analysis was also tested for *Bartonella* spp. identification. From the beginning, the problem with MALDI-TOF use was the low amount of biomass

obtained from the samples. The low biomass yield from passaged clones was caused by long-term cultivation of the original samples before re-inoculation.

For the identification of bacteria by MALDI-TOF analysis, it is recommended that colonies should be as fresh as possible, preferably duration of cultivation of up to 48 hours, although the identification of slow-growing bacteria is also possible (Biswas & Rolain, 2013; Chean *et al.*, 2014; Liébana-Martos, 2018). In our study, the ability to recognize bartonellae from samples and distinguish species decreased from the original 95% to 17%. Samples used for analysis were cultivated for two to six months prior to analysis, which could cause the lack of measurements in the samples. However, the worst results were obtained from samples that were grown for up to three months, not from samples, that were cultivated for six months before analyses. To conclude, the combination of long-term cultivation and a low amount of biomass could lead to a rapid decrease in the recognition rate. To determine the practicality of this method in slow-growing bacteria, further experiments are needed. However, it would be necessary to use fresh samples with a sufficient amount of biomass.

### **6.3.2 Characterization of cultured species and genotypes**

The cultivated passaged clones (3 times) of two species (*B. grahamii*-like and *B. sp. NEW*) represented by two genotypes each were used for further characterization. The morphology of colonies and bacteria corresponds to the characterization of other *Bartonella* spp. (Birtles *et al.*, 1995; Heller *et al.*, 1999; Gutiérrez *et al.*, 2020). The preferred cultivation conditions were tested and the best growth was determined to be 37 °C with 5% CO<sub>2</sub>, the common conditions used for cultivation of other *Bartonella* spp. (Brenner *et al.*, 1993; Gutiérrez *et al.*, 2017). However, comparable growth was observed at 32 °C with CO<sub>2</sub>, although the growth rate was slower. In several studies, the 32 °C cultivation temperature was also successfully tested for other *Bartonella* species, e. g. *B. australis* (from kangaroos), *B. henselae*, and *B. clarridgeiae* (both from cats) (Marston *et al.*, 1999; Fournier *et al.*, 2007).

On the other hand, growth at 42 °C with CO<sub>2</sub> and at 37 °C without added CO<sub>2</sub> was very slow and no colonies or only a very few managed to grow. However, one agar plate of *B. grahamii*-like was capable of growing even at 37 °C without CO<sub>2</sub> and the growth was comparable to the growth observed at 32 °C with CO<sub>2</sub>. The inability of *B. ancahensis* to grow without added CO<sub>2</sub> and at a low temperature of 26 °C was described; however, the optimal cultivation temperature was detected at 30 °C (Mullins *et al.*, 2015). The inability of *Bartonella* spp. to grow at a temperature of 42 °C could be related to the adaptation of bartonellae to the range of temperatures they can encounter during their lifecycle. Although bartonellae can encounter much lower temperatures than 32 °C during their lifecycle (in larvae and pupae stages), they probably survive this period in a dormant stage without any growth or reproduction as was described for *Yersinia pestis* (Wimsatt *et al.*, 2009).

## 7 Conclusions

This study focused on the detection and characterization of bartonellae infection in edible dormice and associated fleas. A total of 204 dormice (*Glis glis*) and 823 *Ceratophyllus sciurorum* fleas (510 removed from dormice and 155 from nests, supplemented with 158 individuals used for different life stages experiment) sampled over six years were tested for *Bartonella* spp. presence. The accuracy and suitability of the protocols chosen for *Bartonella* spp. detection was discussed.

The prevalence of *Bartonella* spp. reached 89%, 90%, and 40% in dormice, fleas from dormice, and nests, respectively. No significant differences were detected between rodent or flea sexes. Four *Bartonella* spp. (two of them in various genotypes) were identified including a new species (*Bartonella* sp. NEW) and subspecies (*B. grahamii*-like = *B. sp. SCIER*), which have not been formally described so far. The high prevalence of these two *Bartonella* species detected in edible dormice and their fleas indicates an important role of this rodent and flea species in their lifecycle.

Possible transmission routes of bartonellae between different life stages of fleas were studied. The flea feces were tested for the presence of viable *Bartonella* spp.; however, the experiment was not successful due to the high contamination rate. Both larvae and newly emerged adult fleas were found to be *Bartonella* PCR-positive, which indicates the vertical transmission via ingesting of infected feces by larval stages and transstadial transmission to newly emerged fleas. However, it is not clear whether the *Bartonella* spp. detected in newly emerged adult fleas are viable because cultivation was not successful. Similar *Bartonella* spp. prevalence in three studied life stages indicates a possible role of fleas in the long-term survival of bartonellae in rodent nests.

A total of 234 dormice and flea samples were cultivated on blood agar plates. The identification of species by MALDI-TOF was tested; however, it was found to be unreliable in the ability to detect data for *Bartonella* spp. identification, probably due to the bacteria slow growth. The two most abundant species, *B. grahamii*-like, and *B. sp. NEW*, were successfully cultivated and subsequently used for phylogenetic and phylogenomic analyses, morphological and growth characterizations, and will be formally described soon.

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