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Chlamydiaceae and chlamydia-like organisms in free-living small mammals in Europe and Afghanistan

Stephan, S ; Guerra, D ; Pospischil, A ; Hilbe, Monika ; Weissenböck, H ; Novotny, L ; Greub, Gilbert ; Croxatto, A ; Teifke, J P ; Ulrich, R G ; Schlegel, M ; Ruhl, S ; Schotte, U ; Binder, A ; Sauer, S ; Borel, Nicole

Abstract: Few data are available on the occurrence of chlamydial infections in wild small mammals. Therefore, the present study aimed to investigate the significance of free-living small mammals as reservoirs or transmission hosts for chlamydiae. In total, 3,664 tissue samples originating from 911 animals were collected in Switzerland, Germany, Austria, the Czech Republic and Afghanistan. Samples included internal organs (n = 3,652) and feces (n = 12) from 679 rodents (order Rodentia) and 232 insectivores (order Eulipotyphla) and were tested by three different TaqMan real-time polymerase chain reactions (PCR) specific for members of the family Chlamydiaceae and selected Chlamydia-like organisms such as Parachlamydia spp. and Waddlia spp. Only one out of 911 (0.11%) animals exhibited a questionable positive result by Chlamydiaceae specific real-time PCR. Furthermore, five out of 911 animals (0.55%) were positive by specific real-time PCR for Parachlamydia spp. but could not be confirmed by Parachlamydia acanthamoebae secY qPCR. One out of 746 animals (0.13%) showed a positive result by real-time PCR for Waddlia chondrophila. This result was confirmed by Waddlia secY qPCR. This study represents the first detection of Chlamydia-like organisms in small wildlife in Switzerland. Considering previous negative results for Chlamydiaceae in wild ruminant species from Switzerland, these data suggest that wild small mammals are unlikely to be important carriers or transport hosts for Chlamydiaceae and Chlamydia-like organisms.

DOI: <https://doi.org/10.7589/2013-08-194>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-87383>

Journal Article

Accepted Version

Originally published at:

Stephan, S; Guerra, D; Pospischil, A; Hilbe, Monika; Weissenböck, H; Novotny, L; Greub, Gilbert; Croxatto, A; Teifke, J P; Ulrich, R G; Schlegel, M; Ruhl, S; Schotte, U; Binder, A; Sauer, S; Borel, Nicole (2014). Chlamydiaceae and chlamydia-like organisms in free-living small mammals in Europe and Afghanistan. Journal of Wildlife Diseases, 50(2):195-204.

DOI: <https://doi.org/10.7589/2013-08-194>

1 Stephan et al. – Chlamydiae in wild small mammals.

2 **OCCURRENCE OF *CHLAMYDIACEAE* AND *CHLAMYDIA*-LIKE ORGANISMS IN**
 3 **FREE-LIVING SMALL MAMMALS IN EUROPE AND AFGHANISTAN**

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34 Word count: Abstract: 220; Introduction –Discussion: 3,108

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51 **ABSTRACT**

52 Few data are available on the occurrence of chlamydial infections in wild small
53 mammals. Therefore, the present study aimed to investigate the significance of free-living
54 small mammals as reservoirs or transmission hosts for chlamydiae. In total, 3,664 tissue
55 samples originating from 911 animals were collected in Switzerland, Germany, Austria, the
56 Czech Republic and Afghanistan. Samples included internal organs (n = 3,652) and feces (n
57 = 12) from 679 rodents (order Rodentia) and 232 insectivores (order Eulipotyphla) and were
58 tested by three different TaqMan real-time polymerase chain reactions (PCR) specific for
59 members of the family *Chlamydiaceae* and selected *Chlamydia*-like organisms such as
60 *Parachlamydia* spp. and *Waddlia* spp. Only one out of 911 (0.11%) animals exhibited a
61 questionable positive result by *Chlamydiaceae* specific real-time PCR. Furthermore, five out
62 of 911 animals (0.55%) were positive by specific real-time PCR for *Parachlamydia* spp. but
63 could not be confirmed by *Parachlamydia acanthamoebae* secY qPCR. One out of 746
64 animals (0.13%) showed a positive result by real-time PCR for *Waddlia chondrophila*. This
65 result was confirmed by *Waddlia* secY qPCR. This study represents the first detection of
66 *Chlamydia*-like organisms in small wildlife in Switzerland. Considering previous negative
67 results for *Chlamydiaceae* in wild ruminant species from Switzerland, these data suggest that
68 wild small mammals are unlikely to be important carriers or transport hosts for
69 *Chlamydiaceae* and *Chlamydia*-like organisms.

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71 **Key words:** *Chlamydiaceae*, *Parachlamydia*, Real-time PCR, *Waddlia*, wildlife

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75 INTRODUCTION

76 Chlamydiae are an important group of obligate intracellular microorganisms which
77 cause a variety of diseases in mammals and birds (Longbottom and Coulter, 2003).
78 *Chlamydia muridarum*, the agent of the so-called Mouse Pneumonitis (MoPn), was
79 previously classified as a biovar of the species *Chlamydia trachomatis*. It is composed of
80 two strains, the Nigg strain (MoPn) and the SFPD strain (Zhang et al., 1993; Everett et al.,
81 1999). Both have been isolated from laboratory mice and hamsters (Nigg, 1942; Stills et al.,
82 1991). While *C. trachomatis* infects humans, the closely related *C. muridarum* species
83 naturally occurs in members of the family *Muridae*, producing a subclinical respiratory
84 infection in young laboratory Albino Swiss mice (Nigg and Eaton, 1944). *C. muridarum*
85 (MoPn) infection in laboratory mice was observed for the first time by Dochez et al. in 1937.
86 Moreover, as all oculo-genital strains of *C. trachomatis* are able to infect mice when
87 inoculated with highly infectious material (Storz and Page, 1971), laboratory mice became a
88 widely used animal model for the investigation of human chlamydial infections (Laitinen et
89 al., 1997). Strikingly, there is no report documenting isolation of *C. muridarum* from wild
90 rodents. In addition, no investigator has ever tried to isolate chlamydiae from wild
91 insectivores such as shrews, hedgehogs and moles.

92 Mouse models are widely used to provide insight into the pathogenesis of *Chlamydia*
93 *abortus*, the agent of ovine enzootic abortion (OEA) because mice show the same clinical
94 signs (abortion and pneumonia) as those observed in small ruminants (Caro et al., 2009). In
95 Switzerland, seroprevalence in small ruminants was highest (43%) in the Canton of Grisons
96 (Borel et al., 2004), where even wild ruminants are sporadic carriers of this abortigenic agent
97 (e.g., Holzwarth et al., 2011a,b). As interactions between domestic or wild ruminants and
98 free-living small mammals may occur on Alpine pastures, the role of the latter as reservoirs
99 of *C. abortus* and other *Chlamydiaceae* should be considered.

100 In the present survey, we also concentrated on a search for *Chlamydia*-like organisms
 101 *Parachlamydia acanthamoebae* and *Waddlia chondrophila*. Both are considered to be
 102 important emerging pathogens in animals with zoonotic potential (Greub and Raoult, 2002).
 103 They might be regarded as new abortigenic agents in Swiss and Scottish cows (e.g., Ruhl et
 104 al., 2009; Deuchande et al., 2010). Moreover, *P. acanthamoebae* has been associated with
 105 ocular lesions in naturally infected guinea pigs (Lutz-Wohlgroth et al., 2006) and cats
 106 (Richter et al., 2010) and was recently shown to produce pneumonia in an experimental
 107 murine lung infection model (Casson et al., 2008b). Furthermore, *Parachlamydia* and
 108 *Waddlia* DNA has been detected in different ruminant wildlife species in Switzerland
 109 (Regenscheit et al., 2012) and even in environmental samples such as cattle drinking- and
 110 well water (Wheelhouse et al., 2011; Codony et al., 2012). The potential of wild small
 111 mammals being a source of *Chlamydia*-like organisms has so far not been investigated.

112 Therefore, the aim of the present study was to elucidate the occurrence of
 113 *Chlamydiaceae*, with focus on *C. muridarum* and *C. abortus* as well as selected *Chlamydia*-
 114 like organisms such as *P. acanthamoebae* and *W. chondrophila* in free-living small
 115 mammals of different geographical regions in the Old World such as Switzerland, Germany,
 116 Austria, the Czech Republic and Afghanistan.

117 MATERIALS AND METHODS

118 In total, 3,652 tissue samples and 12 fecal samples out of 911 wild small mammals of
 119 20 different species were available from Switzerland (n = 490), Afghanistan (n = 379),
 120 Germany (n = 29), Austria (n = 8) and the Czech Republic (n = 5) (Table 1). Animals
 121 (n = 490) from Switzerland originated from different Swiss cantons: Grisons (n = 277),
 122 Lucerne (n = 201), Aargau (n = 8), Fribourg (n = 3) and Zurich (n = 1). Samples from the
 123 canton of Grisons were available from two recent studies investigating shrews as a reservoir
 124 host of Borna Disease Virus (Hilbe et al., 2006; Puorger et al., 2010). Animals originating

from the canton of Lucerne were available as part of a collaborative project with the Institute of Veterinary Parasitology, University of Zurich and were obtained in August and November 2012. Samples from other Cantons were provided by private individuals. All animals were trapped in live or killing traps and for each captured animal details including species, sex, age and origin were recorded. Small mammals originating from Afghanistan (n = 379) were trapped in military camps of the International Security Assistance Force (ISAF) in Mazar-e-Sharif (n = 302), Kunduz (n = 51) and Fayzabad (n = 26), between November 2010 and March 2011 and necropsied according to standard protocol (Schlegel et al., 2012a). For the present study, DNA preparations of the kidney and the liver were used. Additionally, small mammals from Germany (n = 29) and Austria (n = 8) were collected by the Department of Pathobiology, University of Veterinary Medicine, Vienna. Mice from the Czech Republic (n = 5) originating from the South Moravia Region were provided by the Faculty of Veterinary Medicine, Brno. For animals originating from Germany, Austria and the Czech Republic, no detailed information besides the species and the origin was available. All samples besides those from Afghanistan were archived formalin-fixed and paraffin-embedded (FFPE).

In summary, 3,664 samples from a total of 911 animals were investigated. Gastrointestinal tract samples (n = 688) consisted of small intestine (n = 343), large intestine (n = 330), pancreas (n = 11), oesophagus (n = 3) and stomach (n = 1). Lymphatic tissue (n = 375) included spleen (n = 368) and lymph node (n = 7). Testis (n = 5), epididymis (n = 3), mammary gland (n = 2) and embryo (n = 2) were included as reproductive tract samples (n = 12). The species determination of all animals from Afghanistan (n = 379) was performed according to recently published protocols (Schlegel et al., 2012b). Species determination of animals positive or questionable positive for chlamydiae (Table 2) was performed by PCR protocols according to Schlegel et al., (2012b) and revealed a distinct sequence for the shrew positive for *Waddlia* spp. (*Neomys anomalus*).

Sections of 30 µm of formalin-fixed, paraffin-embedded tissue samples were deparaffinized in xylene. After centrifugation at 13,500 g for 5 min, the xylene was removed by repeated extraction in ethanol followed by a second centrifugation and the removal of residual ethanol (95%). The pellet was treated overnight with proteinase K (20 mg/ml, Roche Diagnostics GmbH, Mannheim, Germany) on a thermomixer (55°C, 550 rpm). The DNA was extracted using a commercial DNeasy Blood Tissue Kit and QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The DNA concentration measurement was performed for each sample by measuring the absorbance at A260 nm and A260/280 absorbance ratio respectively on a spectrophotometer Nanodrop® 1000 Version 3.7.1. (Thermo Fisher Scientific, Wilmington, USA). DNA content ranged from 1.1 to 821 ng/µl and showed an average value of 226 ng/µl. The OD (optical density) 260/280 ranged from 1.69 to 1.98 and showed an average value from 1.87.

All samples were examined in duplicate on an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA) using a 23S-rRNA gene-based *Chlamydiaceae* family-specific real-time PCR as described recently (Ehrlich et al., 2006). Primers and probe (Microsynth, Balgach, Switzerland) were used as followed to amplify a 111-bp product specific for members of the *Chlamydiaceae*: Ch23S-F (5'-CTGAAACCAGTAGCTTATAAGCGGT-3'), CH23S-R (5'-ACCTCGCCGTTTAACTTAACTCC-3') and Ch23-p (FAM-CTCATCATGCAAAAGGCACGCCG-TAMRA). Furthermore, an internal amplification control consisting of primers EGFP-1-F (5'-GACCACTACCAGCAGAACAC-3'), EGFP-10-R (3'-CTTGTACAGCTCGTCCATGC-5') and probe EGFP-HEX (HEX-AGCACCCAGTCCGCCCTGAGCA-BHQ1) to generate a 177-bp product, was included. If the internal control was inhibited, the sample was retested at a 1:10 dilution. To yield a final

175 volume of 25 µl, 2.5 µl of DNA template was added to a mix of reagents containing 12.5 µl
176 of 2xMasterMix-buffer (TaqMan® Fast Universal PCR Master Mix (Applied Biosystems,
177 Darmstadt, Germany) and a final concentration of 500 nM of each primer and probe
178 (Microsynth). The cycling profile included initial denaturation at 95°C for 10 minutes,
179 followed by 45 cycles of denaturation and amplification at 95°C for 15 seconds and 60°C for
180 30 seconds. An automatically calculated cycle threshold value (Ct value) of < 38 was
181 considered as positive. When both Ct-values were < 38, a sample was considered as positive.
182 If only one Ct-value of < 38 or a mean Ct-value of > 38 was obtained, a sample was
183 considered as questionable positive. Questionable positive samples were tested again in
184 duplicate by the same method. Samples without a Ct-value were interpreted as negative. *C.*
185 *abortus* DNA was used as a positive control whereas the negative control consisted of a
186 reaction mix with water (H₂O).

187 Samples with at least two Ct-values were further examined by the species-specific 23S
188 ArrayTube (AT) Microarray assay (Alere Chip Technologies GmbH, Jena, Germany) as
189 described recently (Borel et al., 2008).

190 All *Chlamydiaceae* real-time PCR questionable positive samples were further
191 investigated in duplicate by a 16S-rRNA-based *Chlamydiales*-specific real-time PCR, which
192 should result in the generation of a 207-215 bp PCR product (variability in length depends
193 on the species) (Lienard et al., 2011). A final volume of 20 µl was obtained by adding a
194 commercial mastermix (Bio-Rad, Reinach, Switzerland), 0.1 µM concentrations of each
195 primer and probe (Eurogentec, Seraing, Belgium), molecular-biology-grade water (Sigma-
196 Aldrich, Buchs, Switzerland), and 5 µl of DNA sample. The PCR run started with an initial
197 denaturation (95°C, 3 min), followed by 50 cycles of denaturation, annealing and extension
198 (95°C, 15 sec; 67°C, 15 sec; 72°C, 15 sec).

199 Tissue samples (n = 3,422) out of 911 animals were examined with an ABI Prism 7500

instrument (Applied Biosystems, Foster City, CA, USA) applying a modified version of a real-time PCR for the specific detection of *Parachlamydia* spp. as described by Casson et al., 2008a). This PCR assay should result in a 103-bp product of the 16S-rRNA gene specific for *Parachlamydia* spp. To yield a final volume of 25 µl, 0.5 µl of primer PacF (5'-CTCAACTCCAGAACAGCATTT-3'), 0.5 µl of primer PacR (5'-CTCAGCGTCAGGAATAAGC-3') and 0.25 probe PacS (5'-tetrachloro-6-carboxyfluoresceinTTCCACATGTAGCGGTGAAATGCGTAGATATG-Black Hole Quencher 1-3') (Applied Biosystems, Darmstadt, Germany) each with a final concentration of 10 µM was added to a reaction mix of 2.5 µl DNA sample and 12.5 µl iTaqSupermix® (Bio-Rad, Rheinach, Switzerland). The cycling conditions were 3 minutes at 95°C for initial denaturation, followed by 45 cycles of denaturation and amplification for 15 seconds at 95°C and 1 minute at 60°C. Samples were tested at least in duplicates and were considered negative if no amplification was observed during all 45 cycles (Blumer et al., 2011).

For *Waddlia* specific DNA amplification, the cycling conditions were the same as for *Parachlamydia*. Primers WadF, WadR and probe WadS were used as described by Goy et al., (2009) to generate a 101-bp product within the 16S-rRNA gene specific for *Waddlia* spp. In total, 746 samples were tested using this PCR assay. A plasmid containing a part of the 16S gene sequence of *Parachlamydia* spp. or *Waddlia* spp. was used as a positive control, respectively. As negative control, a reaction mixture with water (H₂O) was amplified. Samples were tested at least in duplicates and were considered negative if no amplification was observed during all 45 cycles (Blumer et al., 2011).

All samples positive or questionable positive with the pathogen specific PCR targeting the 16S-rRNA gene were confirmed by PCR targeting the secY encoding gene of *P. acanthamoebae* and *W. chondrophila*, respectively. To yield a final volume of 20 µl, 0.4 µl

(200nM) of primer secY_Parachlam_F2 (5'-GCTCTTTGGCCATCTCTACAGCGT-3'), 0.4 µl (200nM) of primer secY_Parachlam_R2 (5'-CCAGCGACGATACCTGGCTTTGA-3') and 0.2 µl (100nM) of probe secY_Parachlam_S2 (5'-FAM-ACGCGAAAATGGGGAAGCGGCAAAGCG-BHQ1-3') were added to a reaction mix of 5 µl DNA sample and 10 µl of iTaqSupermix with ROX (BioRad). In the same manner, primers and probe for *W. chondrophila* were used as followed: secY_Wad_F1 (5'-CGCCAAGGGCGTCCAACCTCA-3'), secY_Wad_R1 (5'-AGCAAAGCCGTACCGCCAAAGA-3'), secY_Wad_S1 (5'-FAM-ACTCTCATCGGCGCGGTTTTCTTGCT-BHQ1-3'). Seven tissue samples were examined in duplicate with a StepOne plus (Life Technologies, Carlsbad, CA, USA) instrument. The cycling conditions were the same as for the 16S-rRNA PCR for *Parachlamydia* spp. or *Waddlia* spp., respectively. Both species-specific secY PCR assays amplify a DNA region of about 100 bp.

RESULTS

Of 3,664 samples from 911 animals, one organ sample consisting of the small and large intestine from one vole (0.11%) was questionable positive by real-time PCR for *Chlamydiaceae* with a mean Ct value of 40.1 (Table 2). This result could not be confirmed by the *Chlamydiales* 16S-rRNA PCR and species identification by ArrayTube Microarray was not conclusive.

Thirteen tissue samples (liver: n = 3, lung: n = 2, heart: n = 2, kidney: n = 2, spleen: n = 2, feces: n = 2) from five animals (0.55%) were positive by real-time PCR for *Parachlamydia* spp. with mean Ct values between 38.8-42.6. However, results of these thirteen samples could not be confirmed by the *Parachlamydia* secY qPCR.

In total, samples of 746 animals were available to test for *Waddlia*. Therefrom, the organ pool consisting of liver, lung heart and kidney from one animal

(0.13%) had a mean Ct value of 38.9 by real-time PCR for *Waddlia* spp. This result was confirmed by *Waddlia* secY qPCR (Ct value 39.0).

DISCUSSION

In total, samples of 679 rodents (Rodentia) as well as 232 insectivores (Eulipotyphla) including shrews, hedgehogs and moles were investigated (Table 1). To the author's knowledge, the current study is the first large-scale screening for *Chlamydiaceae*, and in particular for *C. muridarum*, in wild rodent and insectivore species in different European countries and Afghanistan. DNA of *C. muridarum* was not detected in the investigated wild mammals. Previous studies in laboratory mice indicate that a low level infection without overt disease might be common (Karr, 1943). However, a transient infection of young animals could also be possible (Fox et al., 2006). In the present study, transient infection might not have been detected as samples were only available at a particular time point. Since all 688 organ samples of the gastrointestinal tract were negative by *Chlamydiaceae*-specific real-time PCR, wild small mammals are probably not carriers of intestinal chlamydiae or at least the prevalence of infection is very low. Despite this, it is also possible, that wild small mammals do not normally harbour *C. muridarum*. However, a recent preliminary study has detected chlamydial infection in a New World cricetide rodent, i.e., *Peromyscus* spp. (K. W. Ramsey et al., poster presentation, Fifth Biennial Meeting of the *Chlamydia* Basic Research Society, 2011, Redondo Beach, California, USA).

Interestingly, in an adult water vole captured in the canton of Grisons, where *C. abortus* is endemic in small ruminants (Borel et al., 2004), a questionable result was obtained in the *Chlamydiaceae* real-time PCR but was negative for *C. abortus*. However, the absence of *C. abortus* in the current study is in line with recent surveys in wild ruminants from Switzerland including ibex (4/412 positive), red and roe deer (all 163 negative) and chamois (1/79 positive) tested by the same methods (Holzwarth et al., 2011a,b; Regenscheit et al., 2012).

275 All investigated individuals were also negative for *Chlamydiaceae* other than *C.*
 276 *muridarum* and *C. abortus*. These findings indicate that neither wild ruminant species nor
 277 free-ranging rodent and insectivore species act as significant reservoir or transport host for
 278 *C. abortus* at least in the examined geographical regions. Previous studies in the literature
 279 often rely on serological methods of low sensitivity and specificity such as the complement
 280 fixation test (CFT) (Wilson et al., 2009). The CFT is well known for cross-reacting with
 281 other chlamydial species, which may have led to false positive results (Griffiths et al., 1996).
 282 However, in the present study, sensitive and specific direct TaqMan real-time PCR methods
 283 were carried out on formalin-fixed, paraffin-embedded organ and fecal samples.

284 The present study revealed thirteen samples including internal organs such as liver,
 285 lung, heart, kidney, spleen and feces from five animals out of 911 (0.55%) positive by real-
 286 time PCR for *Parachlamydia* spp. but negative by the *Parachlamydia* secY qPCR. Despite
 287 the restricted availability of GenBank deposited *Chlamydiales* secY sequences, the
 288 alignment of several *Chlamydiales* secY sequences indicate that *P. acanthamoebae* secY
 289 quantitative PCR is specific at the species level (Figure 1). Only one mismatch was detected
 290 between the strains *Parachlamydia acanthamoebae* Hall coccus and UV-7 which should not
 291 prevent PCR amplification. The secY alignment suggests that the quantitative secY PCR is
 292 more specific than the 16S-rRNA PCR due to reduced sequence conservation of secY genes
 293 compared to 16S-rRNA genes. Thus, these five positive animals detected by the
 294 *Parachlamydia* spp. 16S-rRNA real-time PCR but negative by the *P. acanthamoebae* secY
 295 PCR may be infected by a species related to *P. acanthamoebae* but exhibiting enough
 296 difference in the secY target to prevent PCR amplification. Under experimental conditions,
 297 parachlamydial infection in laboratory mice was demonstrated by intratracheal inoculation of
 298 *P. acanthamoebae*, producing an acute purulent to interstitial pneumonia with a mortality
 299 rate of 50% within five days (Casson et al., 2008b). Pathogenicity toward mice was also

300 confirmed by another *in vivo* study investigating the role of Toll-like receptors in the sensing
301 of *P. acanthamoebae* (Roger et al., 2010). Despite this, the role of wild small mammals as
302 either reservoir or carrier host of *Parachlamydia* spp. remains unclear since only results with
303 high Ct-values (ranging from 38.8 to 42.3) were obtained.

304 In total, a pooled organ sample (liver, lung heart and kidney) of one animal (0.13%) was
305 positive (Ct value 38.9) by real-time PCR for *Waddlia* spp., and this result was confirmed by
306 the *Waddlia* secY qPCR (Ct value 39.0). This positive result with two different PCR
307 methods targeting different DNA regions, confirm that the shrew may indeed be infected by
308 *Waddlia* spp. To our knowledge, this is the first detection of *Waddlia* in internal organs of a
309 wild Miller's water shrew (*Neomys anomalus*) from Grisons, Switzerland. Scarce data are
310 available on the prevalence of *Waddlia* spp. and its impact in wildlife. Interestingly, a recent
311 study demonstrated the isolation of a novel *Waddlia* species from urine samples of fruit bats
312 (*Eonycteris spelaeae*) in Malaysia (Chua et al., 2005). To date, *W. chondrophila* is
313 considered as a *Chlamydia*-like organism associated with abortion in ruminants (Henning et
314 al., 2002; Dilbeck-Robertson et al., 2003) and humans (Baud et al., 2011). Interestingly, a
315 recent study on spontaneous abortion in women showed, that seven out of 200 women with
316 recurrent miscarriages and two out of 97 serologically positive women for *Waddlia* spp. have
317 had prior contact to rodents (Baud et al., 2007).

318 In summary, considering the results obtained by different real-time PCR methods, we
319 conclude that *Chlamydiaceae* are absent or occurring very rarely in free-living rodents and
320 insectivores, at least in the populations that we screened. Similarly, these small wild
321 mammals may be only sporadically infected with *Parachlamydia* spp. and *Waddlia* spp.
322 However, further studies in other geographical areas investigating other small wild rodent
323 and insectivore species are needed to shed light on the potential pathogenicity, distribution
324 and transmission of chlamydiae in wildlife populations of underestimated importance.

325 ACKNOWLEDGEMENTS

326 We thank Robert V. Schoborg, from the Department of Microbiology, Quillen College
 327 of Medicine, ETSU, Johnson City, Tennessee for help with manuscript preparation. We are
 328 grateful to Jürg Paul Müller of the Natural History Museum of the Grisons for support in
 329 species identification of small mammals. Many thanks to F. Burach, F. Ehrensperger, M.
 330 Puorger, S. Ruegg and T. Sydler for their assistance in sample collection. The authors are
 331 grateful to the laboratory technical staff (in particular Carmen Kaiser) of the Institute of
 332 Veterinary Pathology, Vetsuisse Faculty, University of Zurich, for technical help. Many
 333 thanks to Sébastien Aeby from the Microbiology Institute of the University of Lausanne for
 334 performing parts of the PCR examination. We thank P. Deplazes and D. Hegglin for their
 335 collaboration. The development of the secY PCR in G. Greub's group was supported by a
 336 SNSF grant n° 310030-130466.

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Table 1

Details of examined animals (n = 911)

Order	Family	Genus	Species	Number (n)	Origin	
Rodentia	Muridae	Apodemus	Wood mouse (<i>Apodemus sylvaticus</i>)	33	CH	
			Alpine field mouse (<i>Apodemus alpicola</i>)	3	CH	
			Yellow-necked mouse (<i>Apodemus flavicollis</i>)	19	CH	
		Mus	Mus sp.	12	CH	
			House mouse (<i>Mus musculus</i>)	332	AFG	
			Rattus	Rattus sp.	1	AFG
		Cricetidae	Arvicola	Fossorial water vole (<i>Arvicola scherman</i>)	126	CH
			Cricetulus	Gray dwarf hamster (<i>Cricetulus migratorius</i>)	38	AFG
			Microtus	Field vole (<i>Microtus agrestis</i>)	5	CH
	Common vole (<i>Microtus arvalis</i>)	101		CH		
		Myodes	Banc vole (<i>Myodes glareolus</i>)	5, 1*	CZ, CH*	
		Gliridae	Eliomys	Garden dormouse (<i>Eliomys quercinus</i>)	3	CH
Eulipotyphla	Soricidae	Crocidura	White-toothed shrews (<i>Crocidura</i> sp.)	8	AFG	
			Bicolored toothed-shrew (<i>Crocidura leucodon</i>)	20, 19*	CH, D*	
			Greater white-tootheds shrew (<i>Crocidura russula</i>)	1	CH	
		Neomys	Miller’s water shrew (<i>Neomys anomalus</i>)	4	CH	
			Eurasian water shrew (<i>Neomys fodiens</i>)	2	CH	
		Sorex	Long-tailed shrews (<i>Sorex</i> sp.)	83, 8*, 2**	CH, A*, D**	
			Common shrew (<i>Sorex araneus</i>)	41, 8*	CH, D*	
			Eurasian pygmy shrew (<i>Sorex minutus</i>)	14	CH	
			Alpine Shrew (<i>Sorex alpinus</i>)	1	CH	
		Erinaceinae	Erinaceus	European hedgehog (<i>Erinaceus europaeus</i>)	14	CH
		Talpidae	Talpa	European mole (<i>Talpa europaea</i>)	7	CH

CH, Switzerland; D, Germany; A, Austria; CZ, Czech Republic; AFG, Afghanistan

Table 2

Details of seven animals positive or questionable positive by real-time PCR for *Chlamydiaceae*, *Parachlamydia* spp., *Waddlia* spp. and *Chlamydiales*.

Animal	Species	Origin	Sex	Age	Organs	DNA-Concentration ng/ μ m	Real-time PCR for <i>Chlamydiaceae</i> (Ø Ct Value)	16S-base <i>Chlamydiales</i> qPCR (Ø Ct Value)	Real-time PCR for <i>Parachlamydia</i> (Ø Ct Value)	<i>Parachlamydia</i> secY qPCR (Ø Ct Value)	Real-time PCR for <i>Waddlia</i> (Ct Value)	<i>Waddlia</i> secY qPCR (Ø Ct Value)
Vole	<i>Arvicola scherman</i>	Switzerland, Grisons	M	Adult	Small and large intestine	96.3	Questionable positive (40.1)	Negative	Negative	Negative	Negative	Negative
Shrew	<i>Crocidura leucodon</i>	Switzerland, Grisons	NA	Juvenile	Liver, lung, heart, kidney, spleen	71.7	Negative	ND	Positive (38.8)	Negative	Negative	ND
Mouse	NA	Switzerland, Grisons	F	Adult	Feces	10.4	Negative	ND	Positive (39.3)	Negative	Negative	ND
Shrew	NA	Switzerland, Zurich	F	Adult	Feces	68.7	Negative	ND	Positive (40.5)	Negative	Negative	ND
Vole	<i>Microtus arvalis</i>	Switzerland, Grisons	M	Adult	Liver, lung, heart, kidney, spleen	153.7	Negative	ND	Positive (41.6)	Negative	Negative	ND
Mouse	<i>Mus musculus</i>	Afghanistan	NA	NA	Liver	NA	Negative	ND	Positive (42.3)	Negative	Negative	ND
Shrew	<i>Neomys anomalus</i>	Switzerland, Grisons	M	Adult	Liver, lung, heart, kidney	136.4	Negative	ND	Negative	ND	Positive (38.9)	Positive (39.0)

NA: not available; ND: not done; F: female; M: male

459 **Figure 1.** Alignment of the primers and probes used for quantitative real-time PCRs
 460 targeting the *secY* genes of *Parachlamydia* and *Waddlia* spp. (A) Nucleotide sequence
 461 alignment of *Chlamydiales* *secY* gene regions targeted by the *secY* quantitative real-time
 462 PCRs of *Parachlamydia acanthamoebae* strain Hall coccus. Primers (*secY* Para-For and
 463 *secY* Para-Rev) and probe (*secY* Para-S) of the quantitative real-time PCR are indicated by
 464 purple arrows. Highlighted bases represent mismatches to the reference sequence of
 465 *Parachlamydia acanthamoebae* strain Hall coccus (B) Nucleotide sequence alignment of
 466 *Chlamydiales* *secY* gene regions targeted by the *secY* quantitative real-time PCRs of
 467 *Waddlia chondrophila*. Primers (*secY* Wad-For and *secY* Wad-Rev) and probe (*secY* Wad-
 468 S) of the quantitative real-time PCR are indicated by purple arrows. Highlighted bases
 469 represent mismatches to the reference sequence of *Waddlia chondrophila*.

470

471 **Supplementary Table.** Details of investigated organ and fecal samples of 911 wild small
 472 mammals.

A

B

Figure 1

Supplementary Table

Species	Liver	Kidney	Int. Tract	Lung	Heart	Lymph. Tissue	Repro. System	Feces	Other
Wood mouse (<i>Apodemus sylvaticus</i>)	33	33	70	33	33	38			
Alpine field mouse (<i>Apodemus alpicola</i>)	3	3	6	3	3	3			
Yellow-necked mouse (<i>Apodemus flavicollis</i>)	16	16	35	18	18	18			
<i>Mus</i> sp.	12	12		12	12	11	1	12	
House mouse (<i>Mus musculus</i>)	332	217							
<i>Rattus</i> sp.	1	1							
Fossorial water vole (<i>Arvicola scherman</i>)	126	126	297	121	121	126			4
Gray dwarf hamster (<i>Cricetulus migratorius</i>)	38	12							
Field vole (<i>Microtus agrestis</i>)	5	5	10	5	5	5			
Common vole (<i>Microtus arvalis</i>)	101	101	196	101	101	101			3
Banc vole (<i>Myodes glareolus</i>)	3	2	6	3	2	1			
Garden dormouse (<i>Eliomys quercinus</i>)	3	3		3	3	3			
White-toothed shrews (<i>Crocidura</i> sp.)	8	8							
Bicolored toothed-shrew (<i>Crocidura leucodon</i>)	22	26	27	28	23	11	5		3
Greater white-toothed shrew (<i>Crocidura russula</i>)	1	1		1	1				
Miller's water shrew (<i>Neomys anomalus</i>)	4	4		4	4	2			
Eurasian water shrew (<i>Neomys fodiens</i>)	2	2		2	2				
Long-tailed shrews (<i>Sorex</i> sp.)	80	85	14	84	84	22	5		6
Common shrew (<i>Sorex araneus</i>)	30	39	26	39	38	7			2
Eurasian pygmy shrew (<i>Sorex minutus</i>)	14	14		14	14	12			
Alpine Shrew (<i>Sorex alpinus</i>)	1	1		1	1				
European hedgehog (<i>Erinaceus europaeus</i>)	12	12	1	12	12	12	1		
European mole (<i>Talpa europaea</i>)	7	7		7	7	3			
Total (n = 3,664)	854	730	688	491	484	375	12	12	18

Int.Tract: Intestinal tract; Lymph. Tissue: Lymphatic tissue; Repro. Tract: Reproductive tract; Other: Skeletal muscle, Eye, Skin, Adipose tissue, Salivary gland