Life-long shedding of Puumala hantavirus in wild bank voles (Myodes glareolus)

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The knowledge of viral shedding patterns and viraemia in the reservoir host species is a key factor in assessing the human risk of zoonotic viruses. The shedding of hantaviruses (family Bunyaviridae) by their host rodents has widely been studied experimentally, but rarely in natural settings. Here we present the dynamics of Puumala hantavirus (PUUV) shedding and viraemia in naturally infected wild bank voles (Myodes glareolus). In a monthly capture–mark–recapture study, we analysed 18 bank voles for the presence and relative quantity of PUUV RNA in the excreta and blood from 2 months before up to 8 months after seroconversion. The proportion of animals shedding PUUV RNA in saliva, urine and faeces peaked during the first month after seroconversion, but continued throughout the study period with only a slight decline. The quantity of shed PUUV in reverse transcription quantitative PCR (RT-qPCR) positive excreta was constant over time. In blood, PUUV RNA was present for up to 7 months but both the probability of viraemia and the virus load declined with time. Our findings contradict the current view of a decline in virus shedding after the acute phase and a short viraemic period in hantavirus infection – an assumption widely adopted in current epidemiological models. We suggest the life-long shedding as a means of hantaviruses to survive over host population bottlenecks, and to disperse in fragmented habitats where local host and/or virus populations face temporary extinctions. Our results indicate that the kinetics of pathogens in wild hosts may differ considerably from those observed in laboratory settings.

INTRODUCTION

Hantaviruses (family Bunyaviridae) are three-segmented, negative-stranded RNA viruses transmitted by rodents, insectivores and bats (Guo et al., 2013). More than 50 species (ICTV-approved or tentative) are currently known, and most of them are restrained to a single reservoir host species. Several hantaviruses are human pathogens, causing annually up to 50 000 disease cases worldwide. Pathogenic hantaviruses carried by Old World mice, rats and voles (family Muridae, subfamily Murinae; family Cricetidae, subfamily Arvicolinae) cause haemorrhagic fever with renal syndrome (HFRS), and those carried by New World rats and mice (family Cricetidae, subfamilies Sigmodontinae and Neotominae) cause hantavirus cardiopulmonary syndrome (HCPS) in humans (Jonsson et al., 2010). Puumala hantavirus (PUUV) causes a mild form of HFRS, nephropathia epidemica (NE) in humans (Brummer-Korvenkontio et al., 1980) and accounts for the majority of hantaviral disease incidence in Europe (Vaheri et al., 2013; Vapalahti et al., 2003). The reservoir host of PUUV is the bank vole (Myodes glareolus).

Hantaviruses are transmitted among hosts by direct contact (Bernshtein et al., 1999; Botten et al., 2002; Lee et al., 1981; Padula et al., 2004; Yanagihara et al., 1985) and also indirectly via virus-contaminated excreta (Gavrilovskaya et al., 1990; Kallio et al., 2006; Lee et al., 1981; Nuzum et al., 1988) maintaining their infectiousness outside the host for at least two weeks (Kallio et al., 2006). Inhalation of aerosolized rodent excreta is also considered the main route of infection.
to humans (Hjelle & Glass, 2000; Vapalahti et al., 2010). No apparent symptoms have been reported in rodent hosts due to hantavirus infection, but it may impair host survival in nature (Kallio et al., 2007; Luis et al., 2012; Tersago et al., 2012). The hantavirus infection induces a life-long antibody response where IgG appears in the bloodstream 2 to 3 weeks after infection, but viral RNA and/or infectious virus is found in various tissues, especially in the lungs of chronically infected animals (Botten et al., 2003; Hardestam et al., 2008; Meyer & Schmaljohn, 2000; Schountz et al., 2012).

The duration of hantavirus shedding by reservoir rodents is a critical factor for understanding both the transmission dynamics in host populations and the risk for human infections. Hence, patterns of hantavirus shedding and/or viraemia in the reservoir species have been experimentally studied in numerous host–virus pairs (Billings et al., 2010; Botten et al., 2000, 2002, 2003; Fulhorst et al., 2002; Hardestam et al., 2008; Meyer & Schmaljohn, 2000; Schountz et al., 2012). Many of these studies report a transient viraemia of some days to several weeks (Fulhorst et al., 2002; Hutchinson et al., 1998; Lee et al., 1981; Yanagihara et al., 1985) and suggest only occasional viral shedding after an acute phase of few weeks (Botten et al., 2003; Gavrilovskaya et al., 1990; Hardestam et al., 2008; Lee et al., 1981). This pattern of hantavirus shedding by the host has been adopted in a wide array of mathematical models describing hantavirus transmission among rodents and predicting the risk of human infections (Allen et al., 2009; Sauvage et al., 2007; Wolf, 2004).

However, the current knowledge of hantavirus shedding patterns is, to a large extent, drawn from experimental infections of laboratory-reared animals, whereas only few studies have entailed the dynamics of shedding in naturally infected wild rodents (Bernshtein et al., 1999; Korva et al., 2009; McIntyre et al., 2005; Safronetz et al., 2005, 2008). In contrast to laboratory conditions with ad libitum food and water and thermoneutral temperature, rodents under the limited resources in their natural environment, often in harsh climatic conditions, are subjected to trade-offs between reproductive effort, longevity and immune response (Martin et al., 2008; Mills et al., 2010). These trade-offs may translate to enhanced or prolonged replication of pathogens when resource allocation to immune response is compromised. Indeed, several studies have shown fairly large proportions, i.e. 19–91% (Boone et al., 1998, 2002; Kuenzi et al., 2005; McIntyre et al., 2005; Otteson et al., 1996; Rowe et al., 1995; Safronetz et al., 2005, 2006), of wild hantavirus antibody positive rodents to be RNA viraemic compared with what one might expect from the brief viraemia observed in laboratory studies (Easterbrook & Klein, 2008). For these reasons, we hypothesized that also hantavirus shedding via saliva, faeces and urine could last longer in naturally infected wild rodents than has been suggested by infection experiments in the laboratory. In this study, we present the quantities and prevalence of PUUV shedding and viraemia observed in sequential samples of naturally infected wild bank voles as measured by the presence or PUUV RNA over 5 to 10 month individual follow-up periods.

**RESULTS**

**PUUV RNA presence in excreta**

PUUV was found in saliva, faeces and urine of bank voles for extensive periods after the approximated time of seroconversion; in each excretion, PUUV RNA was present for up to 213 days (±26 days, due to uncertainty of the seroconversion time) after seroconversion (Fig. 1). In some animals, PUUV RNA was also detected long before seroconversion: in saliva, up to 50 (±16) days and in urine, up to 55 (±16) days prior to seroconversion. In faeces, PUUV was first detected 16 (±16) days prior to seroconversion. Overall, PUUV RNA was found in approximately half of the saliva and urine samples, whereas only one-fourth of faecal samples were reverse transcription quantitative PCR (RT-qPCR) positive (Table 1a). In samples taken after seroconversion, 57%, 28% and 51% of saliva, faecal and urine samples were RT-qPCR positive, respectively. The proportion of animals shedding PUUV was highest within

![Fig. 1. The presence and relative quantities of PUUV RNA in sequential samples of saliva (blue), faeces (black), urine (yellow) and blood (red lines) of individual bank voles in relation to seroconversion. Due to uncertainty in the exact time of seroconversion in wild-caught rodents, intervals of approximated time from seroconversion are shown by lines instead of points. The line width illustrates the log_{10} relative quantity of PUUV RNA. Gray lines indicate samples where no PUUV RNA was detected and dashed lines RT-qPCR positive samples where RNA quantification was not performed. Letters M and F refer to males and females.](http://vir.sgmjournals.org)
the first month after seroconversion in all three excreta (Table 1a). Shedding PUUV RNA via any one route appeared to be intermittent; in half of the cases (10/21) where two non-sequential samples of the same animal and excretion showed positive for PUUV RNA, there was a negative sample in between. However, in most of these cases, the animal concurrently shed PUUV via another route or was viraemic (Fig. 1). The bank voles exhibited notable individual variation in temporal patterns of shedding. In some animals, the RT-qPCR-positive samples were concentrated around the point of seroconversion (Fig. 1; M6, M12, F3); in others, positive samples occurred evenly throughout the study period (M4, M5, M7, M11, F2, F5) and the excreta of some animals did not become RT-qPCR positive until a few months after seroconversion (M3, M10). Among the candidate models analysing the probability of excreta being PUUV RNA positive, the best supported model was the one with a single smooth term of time from seroconversion ($P=0.0014$) and different intercepts for each excretion (Table 2). The probability of a bank vole of shedding PUUV RNA in faeces was significantly lower than the probability of shedding in saliva or urine (Wald statistic $P<0.0001$ for saliva and $P=0.016$ for urine). The model predicted the probability of a bank vole of shedding PUUV RNA to peak within the first month after seroconversion in all three excreta (Fig. 2). During the second and third months after seroconversion, the probability of shedding declined, and from 3 to 8 months after seroconversion, it remained on a constant level. The predicted probability of a bank vole of shedding PUUV RNA after 3 months from seroconversion was 71 % of the probability predicted for the first month in saliva, 54 % in faeces and 67 % in urine (Fig. 2). The models including sex or its interaction with time as explanatory variables for PUUV presence in excreta were not supported by Akaike information criteria corrected for sample size (AICc; Hurvich & Tsai, 1989; Table 2).

### Table 1. Bank vole excreta and blood tested and positive for PUUV RNA (a) and the geometric means and 95 % confidence intervals of PUUV RNA relative quantities in RT-qPCR positive samples (b)

Numbers are pooled for each month by the approximated time from seroconversion of each animal. CI, confidence interval; N, total number of samples; –, no data/not applicable.

(a)

<table>
<thead>
<tr>
<th>Months from seroconversion</th>
<th>Saliva</th>
<th>Faeces</th>
<th>Urine</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive/N</td>
<td>%</td>
<td>Positive/N</td>
<td>%</td>
</tr>
<tr>
<td>−2</td>
<td>3/16</td>
<td>19</td>
<td>0/1</td>
<td>0</td>
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</tr>
<tr>
<td>8</td>
<td>1/2</td>
<td>50</td>
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<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>50/100</td>
<td>50</td>
<td>25/98</td>
<td>26</td>
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(b)

<table>
<thead>
<tr>
<th>Months from seroconversion</th>
<th>Saliva</th>
<th>Faeces</th>
<th>Urine</th>
<th>Blood</th>
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</thead>
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<tr>
<td></td>
<td>Mean</td>
<td>95 % CI</td>
<td>Mean</td>
<td>95 % CI</td>
</tr>
<tr>
<td>−2</td>
<td>52</td>
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<td>–</td>
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<td>8</td>
<td>209</td>
<td>–</td>
<td>490</td>
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Table 2. AICc scores of all candidate models analysing the probability and quantity of PUUV RNA shedding and viraemia in bank voles

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Candidate model</th>
<th>Probability</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>Shedding</td>
<td>Separate time smoothers for each excretion + separate intercepts for each sex</td>
<td>315.3</td>
<td>264.7</td>
</tr>
<tr>
<td></td>
<td>Separate time smoothers for each sex + separate intercepts for each excretion</td>
<td>309.6</td>
<td>268.5</td>
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<tr>
<td></td>
<td>One time smoother + separate intercepts for each excretion and sex</td>
<td>300.8</td>
<td>264.0</td>
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<td></td>
<td>Separate time smoothers for each excretion</td>
<td>313.8</td>
<td>261.2</td>
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<td></td>
<td>Separate time smoothers for each sex</td>
<td>320.9</td>
<td>262.5</td>
</tr>
<tr>
<td></td>
<td>One time smoother + separate intercepts for each excretion</td>
<td>299.3*</td>
<td>260.2</td>
</tr>
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<td></td>
<td>One time smoother + separate intercepts for each sex</td>
<td>312.7</td>
<td>256.5</td>
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<td></td>
<td>Separate intercepts for each excretion</td>
<td>308.1</td>
<td>255.2</td>
</tr>
<tr>
<td></td>
<td>Separate intercepts for each sex</td>
<td>311.2</td>
<td>253.3</td>
</tr>
<tr>
<td></td>
<td>Constant quantity through time, excreta and sexes</td>
<td>306.5</td>
<td>251.8</td>
</tr>
<tr>
<td></td>
<td>Separate intercepts for each sex</td>
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<td></td>
<td>Separate intercepts for each sex</td>
<td>316.7</td>
<td>246.4*</td>
</tr>
<tr>
<td>Viraemia</td>
<td>Separate time smoothers for each sex</td>
<td>146.3</td>
<td>97.1</td>
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<tr>
<td></td>
<td>One time smoother + separate intercepts for each sex</td>
<td>138.0</td>
<td>91.0</td>
</tr>
<tr>
<td></td>
<td>One time smoother</td>
<td>135.9‡</td>
<td>88.7§</td>
</tr>
<tr>
<td></td>
<td>Separate intercepts for each sex</td>
<td>157.1</td>
<td>92.9</td>
</tr>
<tr>
<td></td>
<td>Constant quantity through time and sexes</td>
<td>155.0</td>
<td>90.4</td>
</tr>
</tbody>
</table>

'Time smoother' refers to a GAMM smooth term of time (days) to/from PUUV seroconversion.
The AICc scores of the best-supported models are written in bold type.
Variance attributable to random individual-level variation in the best-supported models: *0.64; ‡0.05; §0.45; $0.14.

PUUV RNA quantity in excreta

The highest single quantities of PUUV RNA (1096, 19 953 and 30 903 relative units μg⁻¹ RNA in faeces, urine and saliva, respectively) were observed within the first month after seroconversion (Fig. 2). During the first month, the mean quantity in RT-qPCR positive saliva samples was also highest (Table 1b). In faeces, the quantities during the first month averaged lower than in later months. No temporal trends in PUUV RNA quantity could be seen in urine samples. Among the candidate models analysing the relative quantity of PUUV RNA in RT-qPCR-positive excreta, the model with a constant quantity (164 relative units, 95 % CI: 101–263 units) through time and between excreta and sexes was best supported when random individual variation in shedding quantities was assumed (Table 2). The mean quantities (±SD) of total RNA in saliva, faecal, urine and blood RNA elutions were 70 ± 36, 146 ± 105, 47 ± 22 and 78 ± 27 ng μl⁻¹, respectively.

PUUV in blood

PUUV RNA was detected in bank vole blood from 16 (±16) days before to 199 (±13) days after approximated seroconversion (Fig. 1). Overall, 38 % of blood samples were RT-qPCR positive, and the highest proportion (86 %) of viraemic animals was seen 1 month after the seroconversion (Table 1a). Within 1 month before seroconversion, 13 % of samples were PUUV RNA viraemic, and after seroconversion 52 % of blood samples were RT-qPCR positive. Among the candidate models analysing the probability of blood being PUUV RNA positive, the best-supported model appeared to be the one with a smooth term of time from seroconversion (P=0.0015, Table 2). The model predicted more than 50 % of bank voles to be viraemic between 0.5 and 4 months after seroconversion, after which the probability of viraemia declined to zero by the eighth month after seroconversion (Fig. 2). The highest observed quantity (70 795 relative units) occurred 1 month after seroconversion (Fig. 2), when the mean quantity was also the highest (Table 1b). Among the candidate models analysing the relative quantity of PUUV RNA in RT-qPCR positive blood samples, the model with a declining smooth term of time from seroconversion (P=0.0021) was best supported when random individual variation was assumed (Table 2). The model predicted the PUUV RNA relative quantity to drop from 886 relative units (95 % CI: 525–1495 relative units) 1 month before to 47 relative units (95 % CI: 26–83 relative units) 6 months after seroconversion (Fig. 2). The models including sex or its interaction with time as explanatory variables for PUUV presence or quantity in blood were not supported by AICc (Table 2).

DISCUSSION

Hantavirus infection experiments performed on laboratory-reared rodent hosts have shown a few-week acute phase of intensive virus shedding followed by a chronic
phase of absence or occasional shedding in excreta (Botten et al., 2003; Gavrilovskaya et al., 1990; Hardestam et al., 2008; Lee et al., 1981). Our results suggest that this two-phase propagation pattern may not be applicable to describe hantavirus transmission in natural settings, as we showed notably longer periods of PUUV shedding and viraemia to be common in wild, naturally infected bank voles. The proportion of shedding individuals was highest during the first month after seroconversion, but declined by less than half during later months of infection. On the other hand, no statistically significant temporal patterns could be distinguished in the quantity of shed virus. However, in contrast to shedding patterns via excreta, both the proportion of viraemic individuals and the viral load in

Fig. 2. The probabilities of bank vole excreta and blood being PUUV RNA positive (left panel) and log_{10}-transformed relative PUUV RNA quantities in RT-qPCR positive samples (right panel) in relation to the estimated time of seroconversion. Solid lines indicate the model predictions and dashed lines their 95% confidence intervals. Circles denote the observed data. Circle sizes in the left panel indicate the number of overlapping observations (rounded to the nearest 10 days for visual clarity).
blood declined significantly by time from infection. Also in saliva, but not in faeces or urine, the relative quantities of PUUV RNA showed a decreasing trend, but this tendency could not be statistically distinguished from overall individual-level variation. The number of saliva samples studied here (N=50) may have been insufficient to show any temporal trends. However, a decreasing trend of PUUV quantity in blood was statistically supported despite the lower number of blood samples (N=43) in the analysis.

Bank vole saliva and urine were significantly more often PUUV RNA positive than faeces. The lower proportion of positive faecal samples compared to saliva and urine can, to a large extent, be explained by the lower sensitivity of RT-qPCR in faecal samples. Bank vole faeces include PCR-inhibitory components that reduce the sensitivity of the RT-qPCR, so that a large proportion of faecal samples were thus likely false-negative in RT-qPCR (Hardestam et al., 2008). Also, comparison of PUUV concentrations in different excreta is further complicated by the different volumes of excreta in original samples, and the variation of concentrations of total RNA between excreta. In addition, different shedding routes most likely yield varying amounts of viral particles that end up in contact with new, susceptible hosts; most likely, viral particles in saliva are rather effective administered into the bloodstream via a bite wound, but only a low proportion of aerosolized viral particles in a urine or a faecal excretion end up in the nostrils or mouth of susceptible animals.

The long shedding period in wild hosts evidenced here may result from the limited amount of resources available for immune responses in natural conditions. Nevertheless, besides the deprived immune response in wild compared to laboratory-reared animals, other factors may also explain the lesser propagation of hantavirus in laboratory studies: the impaired infectivity of cell-adapted hantavirus for the natural host (Lundkvist et al., 1997), higher than natural infecting doses (Hardestam et al., 2008) and the low immunogenetic variability in colonized experimental animals (Guivier et al., 2010a) may all bias the shedding patterns observed in infection trials.

We acknowledge that the presence of PUUV RNA in blood or excreta does not necessarily indicate infectiousness to new hosts. With this in mind, we did attempt virus isolation from PUUV RNA positive excreta, but all our attempts failed, most likely since no efficient method exists for the isolation of hantviruses, and since our samples were not optimally preserved for virus isolation. However, in cotton rats (Sigmodon hispidus) inoculated with Black Creek Canal hantavirus, infectious virus could be recovered from salivary glands as long as they showed positive for viral cRNA, and the titres of cell culture infectious virus in blood were consistent with the copy number levels of genomic S segment RNA (Hutchinson et al., 1998). Furthermore, PUUV RT-PCR positive saliva, urine and faeces of experimentally infected bank voles proved infectious when administered intranasally to naïve bank voles (Hardestam et al., 2008).

Although not all PUUV RNA detected in rodent samples is necessarily associated within infectious particles, we have no reason to assume that the proportion of infectious virus from detected PUUV RNA would change by time from infection.

Interestingly, we found PUUV RNA in saliva and urine of infected voles as early as 7 weeks (or 5 to 9 weeks, taking into account the sampling interval) before seroconversion. By contrast, following subcutaneous or intramuscular inoculations in laboratory, hantavirus antibodies have been reported to appear already 1 to 3 weeks post infection (Botten et al., 2000; Fulhorst et al., 2002; Gavrilovskay et al., 1990; Hutchinson et al., 1998; Yanagihara et al., 1985). The relatively late appearance of antibodies could to some extent reflect the lower sensitivity of IFAT compared to ELISA (Lundkvist et al., 1991) used for antibody detection in some studies, but may also result from relatively low virus doses in bank vole excreta: in line with our present results, when excreta of PUUV infected bank voles were intranasally administered into colonized bank voles, only one out of seven successfully infected animals seroconverted before 3 weeks post infection (Hardestam et al., 2008).

Thus far, only few data have been published on the occurrence of hantavirus RNA in the excreta of wild rodents (Korva et al., 2009; McIntyre et al., 2005; Safronetz et al., 2005, 2008). Our results of overall PUUV RNA positivity of 57% in saliva and 52% in urine collected from seropositive animals are similar to the observations where 53% and 35% of wild Bayou hantavirus seropositive Oryzomys palustris shed viral RNA in saliva and urine, respectively (McIntyre et al., 2005). By contrast, lower proportions of shedding (7–21% in saliva and 7–18% in urine) have been reported for Sin Nombre hantavirus (SNV) seropositive deer mice (Peromyscus maniculatus; Safronetz et al., 2005, 2008). The difference between these results may arise from several factors, but it is possible that host species of hantviruses differ in their shedding potential as suggested earlier (Meyer & Schmaljohn, 2000; Padula et al., 2004). The above-mentioned studies on wild rodents have also reported temporal trends of hantavirus shedding: SNV occurred more often in the saliva of recently seroconverted than chronically infected deer mice (Safronetz et al., 2008), and higher quantities of PUUV, Dobrava and Saaremaa hantavirus RNA were detected in the urine bladders of recently seroconverted than chronically infected bank voles, yellow-necked mice (Apodemus flavicollis) and striped field mice (Apodemus agrarius), respectively (Korva et al., 2009). However, in these two studies, the timing of seroconversion was based on IgG antibody avidity, which has been shown to be unreliable in low antibody titres (Varner & Dearing, 2011). In the present study, we were able to determine the time of seroconversion with a more reliable accuracy (±13 to ±24 days), followed individual wild hosts much longer than in previous studies and showed hantavirus shedding to last throughout the typical life span (Innes & Millar, 1994; Prévôt-Julliard et al., 1999) of the rodent host species without drastic decline.
Our finding of high prevalence of hantavirus RNA viraemia in seroconverted bank voles is consistent with several earlier studies on wild rodents (Bagamian et al., 2012; Boone et al., 1998, 2002; Kuenzi et al., 2005; McIntyre et al., 2005; Otteson et al., 1996; Rowe et al., 1995; Safronetz et al., 2005, 2006, 2008). Most individuals were viraemic for four months after seroconversion, which is in concordance with the results of Boone et al. (1998), where SNV RNA viraemia persisted for at least 3 months in wild seroconverted deer mice. An experimental study on SNV transmission in deer mice in outdoor enclosures showed a tendency of highly viraemic animals to more often transmit the infection to new hosts within the first 2 months of infection (Bagamian et al., 2012), suggesting that RNA viraemia correlates with host infectiousness. Our data show that although this might be true within the first months of infection, after 4 months from seroconversion the bank voles shed PUUV in excreta whilst the virus was no longer found in blood. Therefore, host viraemia may not be a good indicator of host infectiousness.

In the present study, no sex differences in likelihood, duration or quantity of PUUV shedding were found. This may be due to the fact that most of the bank voles in this study were sexually immature, overwintering subadults, whereas an earlier study (Hannah et al., 2008) showed sex steroids to mediate sex differences in hantavirus shedding. Here, the first months of infection were concentrated in the winter, the most challenging season in terms of temperature regulation and food resources. It remains a target of our further studies to determine how the more abundant food resources and warmer temperature, and, on the other hand, the possible costs of sex steroids, mediate PUUV shedding during the breeding season.

In cyclic fluctuations of bank vole populations, phases of low density may last for more than a year and are characterized by a low abundance or absence of infected individuals (Kallio et al., 2009; Tersago et al., 2008). We suggest that the persistent PUUV shedding by the individuals that outline population bottlenecks facilitates the reinitiation of PUUV transmission as the population again starts to grow. Although PUUV has been demonstrated to remain infectious in the environment for several weeks (Kallio et al., 2006), this time most probably is not long enough for the virus to survive in areas where susceptible hosts are long absent.

The genomes of both rodent host species and their endemic viruses show notable geographical variation (Asikainen et al., 2000; Defontaine et al., 2005) that may translate into differential host–pathogen relationships (Guivier et al., 2010b). It remains to be studied whether the geographical differences in the balance of virus tolerance/resistance are reflected in the shed quantities of PUUV – if true, such a difference could in part explain the higher incidence of NE in Finland and elsewhere in northern Europe compared to France and temperate Europe in general (Vaheri et al., 2013).

The majority of mathematical models describing rodent–hantavirus interactions and/or predicting human hantavirus disease risk assume two discernible phases of shedding in infected rodents – an acute phase versus a chronic phase (Allen et al., 2009; Sauvage et al., 2007; Wolf, 2004). In contrast to these model assumptions, we found no notable decrease in virus shedding during the course of a rodent’s life. This finding suggests that the abundance of infectious hosts, the most important driver for human hantavirus epidemics, may be underestimated by current mathematical models. Whichever the underlying reasons for the discrepancy between experimental and natural infections, our results stress the importance of studying zoonotic infections in natural settings. We strongly encourage further studies on hantavirus shedding by wild hosts in other virus–host pairs and impose a need to revise the assumptions of infectiousness in hantavirus transmission models, which in turn will provide improved tools to predict the threat of hantavirus infection to public health.

METHODS

Collection of wild rodent samples. The study took place at Konnevessi, Central Finland (62° 34′ N 26° 24′ E), where PUUV is endemic in bank voles (Kallio et al., 2007, 2009; Razzauti et al., 2008). The study site is situated in the hospital district of Central Finland, where mean annual NE incidence is 71/100,000, the fourth highest of the 20 hospital districts in Finland (Makary et al., 2010). The excreta and blood samples were collected on 13 trapping periods from January 2008 to May 2009 at 27 to 48 day intervals. The sample collection was a part of a long-term capture-mark-recapture study, where bank voles were captured on a 5.8 ha trapping grid consisting of 246 trapping stations at 15 m intervals. One Ugglan Special live-trap (Grahnb) was permanently placed on each trapping station under a sheet metal ‘trap chimney’ that enabled access to the traps during the snowy period and provided shelter for captured animals. Each trapping period lasted 3–4 days, during which the traps were checked ten times. Bank voles were individually marked on first capture with subcutaneous PIT tags (Trovan), and their blood, urine, faeces and saliva samples were collected on every trapping period. The animals were bled through the retro-orbital sinus using heparinized capillaries (Hirschmann). Two aliquots of 18 μl were taken, one for RT-qPCR and the other for PUUV immunofluorescent antibody test. Urine samples were collected using plastic buckets that were sterilized between animals by soaking with 2.5% Umonium® Instruments & Equipments (Huckert’s International) for over 5 min, machine-washed (60 °C, 2 min pre-rinse, 4 min wash with detergent, 2 min rinse), sprayed with 70% ethanol and air-dried. The animals were carefully placed at the bottom of a bucket and any urine pools generated were immediately collected with a sterile pipette. Samples that came into contact with the animal’s paws, tail, snout or fur were discarded. Fresh faeces were collected from the bucket or directly from the anus. Saliva was collected using Copan flocked swabs (Copan) that were subsequently placed in 500 μl of Universal Transport Medium (Millipore). All samples were immediately frozen on dry ice and stored later in −80 °C until analyses. All handling procedures of wild bank voles followed the Finnish Act on the Use of Animals for Experimental Purposes (62/2006) and took place with permission from Finnish Animal Experiment Board (license number HY 54-05).

Dating the seroconversion and selection of samples for RT-qPCR. As the exact date of infection could not be determined in
naturally infected animals, we dated the blood and excretion sampling times in relation to seroconversion, i.e. the appearance of PUUV specific antibodies, instead of days post-infection. The presence of PUUV antibodies in each animal on each trapping period was determined from blood samples diluted 1:10 in PBS using an earlier described immunofluorescent antibody test (Kallo-Kokko et al., 2006). Altogether, we sampled blood and excreta from 352 bank voles, from which 109 had a known time of seroconversion. Based on the antibody results, we selected blood and excretion samples of bank vole individuals where the follow-up time was the longest, i.e. animals whose blood and excreta had been sampled at least once before and four times after seroconversion, to be analysed for the presence and quantity of PUUV RNA. Altogether 349 samples from 121 trapping occasions of 18 bank voles (12 males, 6 females) were thus analysed, from which 114 were blood, 100 saliva, 98 faeces and 37 urine. In addition, excreta and blood from six trapping occasions of one PUUV antibody negative female bank vole were used as negative controls. The seroconversion of a bank vole had a uniform probability of having happened on any day between the last seronegative and the first seropositive blood sample, but was approximated to have happened on the date exactly between these two sampling dates. As the sampling interval preceding the first seropositive sample varied from 26 to 48 days, the seroconversion could be dated with an accuracy of ±13 to ±24 days. The selected individuals were wintering non-breeding subadults during most of the study, except for two males (M1, M3) that were sexually mature during the month preceding seroconversion, and three individuals (M7, F1 and F3) that had reached breeding condition when last sampled. The seroconversion took place during fall (September–November) in 16/18 animals.

**Extraction of viral RNA.** Viral RNA was extracted from blood samples by dissolving the 18 μl sample into 450 μl TRIzol (Invitrogen Life Technologies), then mixing with 150 μl chloroform and centrifuging for 15 min at 4 °C, at 12,000 g. RNA extraction was then continued using a QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer’s instructions, so that 140 μl of the water phase formed was mixed with 560 μl Buffer AVL (Qiagen) and 5.6 μl cRNA. For saliva, urine and faeces, only 140 μl of the QIAamp Viral RNA Mini kit was used. The faecal samples (each consisting of one to three faecal pellets) were first homogenized in 500 μl NaCl and centrifuged at 4000 g at 4 °C for 10 min. Then, 140 μl supernatant was mixed with 560 μl AVL buffer with 5.6 μl cRNA. For saliva and urine, 70 μl of sample was mixed with 70 μl NaCl, 560 μl Buffer AVL and 5.6 μl cRNA. The RNA elution was stored at −70 °C until analysis. The concentration of RNA was measured after extraction by NanoDrop (Thermo Fisher Scientific).

**RT-qPCR.** The amount of PUUV RNA in samples was estimated by relative quantification of PUUV copies. One-step real-time RT-PCR was performed with a qScript One-Step Fast MGB qRT-PCR kit, low ROX (Quanta BioSciences) in a Stratagene Mx3005p. An ABI Prism 7900 SDS was used instead of the Stratagene Mx3005p for the analyses of the 17 blood samples from three animals (M3, M8 and M12). The primer and probe sequences were optimized to match the PUUV strain circulating at the trapping site (Razzauti et al., 2008): Fw12 – TCTTGTGAAAACTACTACGAGAAAA, Rev87 – TTCAT-GGCGGTTATATCCCTTT, and the probe39 6-FAM-TGGAAT-GAGTGAGTGTGAC-MGB. Five microlitres of cRNAs were added to a reaction that contained 0.9 μM primers and 0.2 μM probes, and had a total volume of 20 μl. The RT-qPCR was confirmed to be highly specific since RNA samples of other hantaviruses tested (Dobrava, Saaremaa, Hantaan and Tula) were negative as well as samples from non-infected bank voles. The standard curve for relative quantification was calculated from 10-fold dilutions of RNA extracted from a lung sample of a PUUV-infected bank vole captured at the same site in an earlier study (Razzauti et al., 2008). The last dilution of RNA (10⁻⁵) that was consistently positive was set as relative quantity 1. The analytical sensitivity of the assay was determined for the different sample materials (Table S1, available in the online Supplementary Material), and was found to be equivalent for saliva, urine and blood, but lower for faeces. The standard RNA was aliquoted and a new dilution series was added to each RT-qPCR run. All samples were initially screened once to find the positive samples. RNA positive samples were then quantified using triplicate reactions, and the mean quantities of the triplicates were used for further analyses. Reactions where the cycle threshold value deviated by more than one cycle from the other two reactions in the triplicate were regarded as artefacts, e.g. pipetting errors, and thus discarded. As the volumes of saliva and faeces used for RNA extraction were unknown, the quantity of PUUV RNA in each positive excretion and blood sample was calculated as the quantity per 1 μg total RNA.

**Statistical analyses.** PUUV shedding (PUUV in saliva, faeces and urine) and viraemia (PUUV RNA in blood) in bank voles were each studied in separate statistical analyses. Generalized additive mixed models (GAMM; Zuur, 2009) in the gamm4 package (Wood, 2011) for R statistical software (R Development Core Team, 2011) were used to analyse the presence and the quantity of PUUV RNA in blood and excretion samples in relation to time of seroconversion and sex. More than half of the samples were RT-qPCR negative, and thus the relative quantity of PUUV RNA showed a highly zero-inflated distribution. When RT-qPCR-negative samples were excluded, the log₁₀-transformed relative quantities showed a normal distribution. Therefore, we studied both shedding and viraemia with two separate models; one to predict PUUV RNA presence/absence with binomial error distributions and a logit link function, and another to predict the log₁₀-transformed relative quantity of PUUV RNA in RT-qPCR positive samples with Gaussian error distributions and an identity link function.

A set of candidate models with different explanatory variables was constituted for each separate analysis, and AICc scores were compared to find the most parsimonious model in each model set, i.e. the smallest possible set of explanatory variables that explains the variation in the response variable. The model with the lowest AICc score was considered as best supported by the data and used for statistical inference. The candidate model sets analysing the presence and quantity of PUUV in excreta included two ‘beyond optimal’ models and all their submodels: one with separate smooth terms of time from seroconversion for each sex and different intercepts for each excretion (saliva/faeces/urine), and another with separate smooth terms of time for each excretion and different intercepts for each sex. The candidate model sets analysing the presence and quantity of PUUV in blood included a ‘beyond optimal’ model with separate smooth terms of time from seroconversion for each sex, and all possible models nested therein. As wild rodents were expected to show great variation in immunogenetic properties, physiological status and other characteristics that may influence hantavirus replication and persistence (Easterbrook & Klein, 2008), random intercepts attributable to individual variation were included in all candidate models.

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