Spatiotemporal dynamics of Puumala hantavirus in suburban reservoir rodent populations

Alexandre Dobly[1,2]✉, Chloé Yzoard[2], Christel Cochez[3], Geneviève Ducoffre[4], Marc Aerts[5], Stefan Roels[1] and Paul Heyman[3]

[1] Pathology, Orientation and Veterinary Support, Veterinary and Agrochemical Research Centre (CODA/CERVA), Groeselenberg 99, 1180 Brussels, Belgium (aldob@var.fgov.be)
[2] Unit of Social Ecology, CP 231, Université Libre de Bruxelles, Bd du Triomphe, 1050 Brussels, Belgium
[3] Reference Laboratory of Vector-Borne Diseases, Queen Astrid Military Hospital, Bruyn Straat, 1120 Brussels, Belgium
[4] Epidemiology Section, Scientific Institute of Public Health, rue Juliette Wytsman 14, 1050 Brussels, Belgium
[5] Interuniversity Institute for Biostatistics and statistical Bioinformatics, Hasselt University & Katholieke Universiteit Leuven, 3590 Diepenbeek, Belgium

Corresponding author:
Alexandre Dobly
CODA/CERVA
Groeselenberg 99
1180 Brussels
Belgium
Email: alexandre.dobly@coda-cerva.be
Fax: +32 2 379 04 79

Abstract

The transmission of pathogens to susceptible hosts is dependent on the vector population dynamics. In Europe, bank voles (*Myodes glareolus*) carry Puumala hantavirus, which causes nephropathia epidemica (NE) in humans. Fluctuations in bank vole populations and epidemics in humans are correlated but the main factors influencing this relationship remain unclear. In Belgium, more NE cases are reported in spring than in autumn. There is also a higher incidence of human infections during years of large vole populations. This study aimed to better understand the link between virus prevalence in the vector, vole demography, habitat quality and human infections. Three rodent populations in different habitats bordering Brussels city, Belgium, were studied for two years. The seroprevalence in voles was influenced first by season (higher in spring), then by vole density, vole weight (a proxy for age) and capture site but not by year or sex. Moreover, voles with large maximal distance between two captures had a high probability for Puumala seropositivity. Additionally, the local vole density showed similar temporal variations than the number of NE cases in Belgium. These results showed that, while season was the main factor influencing vole seroprevalence, it was not sufficient to explain human risks. Indeed vole density and weight (as well as the local habitat) were essential to understand the interactions in this host-pathogen dynamics. This can in turn be of importance for assessing the human risks.

Keywords

Multi-annual cycles, Density-dependence, Epidemiology, Hemorrhagic Fever with Renal Syndrome, Arvicolinae

Introduction

Hantaviruses (family *Bunyaviridae*) are emerging rodent-borne pathogens that circulate worldwide. They are mainly carried by rodents (Muridae and Cricetidae) and by insectivores (Arai et al. 2007). Remarkably each hantavirus is associated with a (very few) distinct rodent species (Plyusnin et al. 1996). The transmission of hantavirus to non-host rodents is nevertheless documented (Klingström et al. 2002) but host specificity merits further investigation, especially among wild sympatric rodents (e.g. Plyusnina et al. 2011).

Some reservoir and/or vector rodents represent very interesting systems to study because they show multi-annual population cycles which have a complex impact on the quantity of circulating pathogens (Davis et al. 2005; Sauvage et al. 2003). Such population fluctuations are believed to be induced by several factors: climate, weather, food, probably predators, but also infectious agents (Cavanagh et al. 2004; Oli 2003; Stenseth et al. 2003). The effect of hantavirus on such demographic fluctuations is perhaps limited as few detrimental effects were described in the vector species. In the natural reservoirs, hantaviruses establish a life-long, chronic infection, and the host develops a strong neutralizing antibody response against the virus (Chu et al. 1994).

However, in humans, hantaviruses are the etiologic agents of hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and of hantavirus pulmonary syndrome (HPS) in the Americas (Peters & Khan 2002). At least 15 of the 35 described hantavirus serotypes are human pathogens (five of them cause HFRS). Worldwide, about 150,000 cases of hantavirus infection are reported annually, mainly in Asia (Vapalahti et al. 2003). Humans are infected by inhalation of aerosols contaminated with excreta from infected rodents. HFRS symptoms include fever, abdominal and back pain as well as renal dysfunction, sometimes with hemorrhagic manifestations (Vapalahti et al. 2003).

In most of Europe, Puumala hantavirus (PUUV), carried by bank voles, *Myodes* (earlier, *Clethrionomys*) *glareolus*, causes a milder form of HFRS known as nephropathia epidemica (NE). This disease is rarely lethal (less than 0.5% of the cases) but may require an average hospital stay of eight days (Paakkala et al. 2004). NE shows multi-annual epidemics in the human population, which are linked to fluctuations in bank vole population density (Schwarz et al. 2009; Tersago et al. 2011; Vapalahti et al. 2003). Until recently such PUUV epidemics in Western Europe showed multi-annual cycles with peaks every three years (Heyman et al. 2001; Sauvage et al. 2003). Since 2001, the peak

frequency changed to every two years (Heyman et al. 2007). This change is associated with an increasing morbidity of PUUV infection in humans with some unexpected sudden rises (Faber et al. 2011).

Season has also an impact on the relationship between vole presence and the number of NE cases. In northern Europe, more infections are recorded in autumn and winter (Olsson et al. 2003) while in Belgium spring is the peak season (Vapalahti et al. 2003). In Belgium, the NE seasonality could be partly due to an increase in human outdoor activities during springtime but the vole population fluctuations could also play an important role. Indeed bank voles overwinter in mixed-sex groups before dispersing in spring as old individuals (Ylönen and Viitala 1991). Within these groups, the virus is very efficiently transmitted by social interactions (biting and grooming, Escutenaire et al. 2002) but also indirectly by inhalation of virions excreted in infectious saliva, urine or feces. Indeed under adequate conditions PUUV can survive for up to two weeks outside their host (Kallio et al. 2006). Therefore the old bank voles dispersing in spring probably show a high seroprevalence (Escutenaire et al. 2000) and could be critical in the dynamics of PUUV infections in humans.

A second important within-population factor is the different infection probability in female, male and juvenile bank voles. During the reproductive season, adult females are territorial and aggressive but seem to show a lower seroprevalence than males (Escutenaire et al. 2002), which have large home ranges that partially cover several female territories (Bujalska & Grüm 1989). The maternal antibodies protect the juveniles from PUUV infection for up to 80 days. Therefore, during the reproductive season, males should probably show a higher seroprevalence than females while juveniles should have a very low PUUV infection rate. This can have significant effects on the dynamics of the infection (Kallio et al. 2006).

Most PUUV studies were carried out on remote rodent populations (Kallio et al. 2007; Sauvage et al. 2002; Tersago et al. 2008, 2011). This paper reports the monitoring of PUUV seroprevalence in suburban populations, which are probably limited in emigration by a highly fragmented and disturbed habitat. The populations were located at close range of heavy human presence, namely the city of Brussels (1.2×10^6 inhabitants). Temporal and spatial dynamics of rodent and virus populations were analyzed to improve the understanding of their impact on human infection risk. For that purpose, factors that could influence PUUV seroprevalence in voles were analyzed: year, season, capture site, vole density, vole age (estimated from weight), vole sex and vole home range size. Two main

assumptions were tested. Since, in Belgium, more human infections are observed in spring than in autumn, it was first checked whether the higher proportion of older voles found in spring would be linked to a higher seroprevalence in spring than in autumn. As the way by which habitat quality influences the virus presence in natural populations is poorly known (Heyman et al. 2009), the effect of weight and home range size on seroprevalence was tested. In addition, the relation between vole PUUV seroprevalence and the number of NE cases in Belgium was examined.

Materials and methods

Sampling and diagnostics

Three sites were sampled in the Sonian Forest along the southern border of Brussels. This forest presents many footpaths for recreational use. Sites 1 and 2, were separated by 500 m and a dense traffic 4-lane road (50°49'N, 4°27'E). Site 1 was located 200 m from private gardens and was frequently visited by walkers. Site 2 was surrounded by areas with no vegetation cover on the ground and thus not favorable to rodents. Site 3 was located 6 km southwest from site 1 in a more remote area with fewer and distant paths. It was not visited by people. On all sites the dominant trees were *Fagus sylvatica*. In site 1 the ground vegetation was in order of abundance *Luzula sylvatica*, *Rubus fruticosus*, *Juncus effuses*, *Deschampsia cespitosa*, *Senecio ovatus*, *Carex pilulifera*, *Deschampsia flexuosa* and *Veronica montana*. Site 2 presented *Hyacinthoides non-scripta*, *Anemone nemorosa*, *Convallaria majalis*, *Lonicera periclymenum* and *Pteridium aquilinum*. At site 3, about 10% of *Quercus robur* was observed with the ground covered by *Rubus fruticosus*.

For two years each site was sampled in autumn and spring for two weeks. The trapping sessions began in September 2003, May 2004, September 2004 and May 2005. Each time a zone of 0.70 ha was sampled with a 10 x 6 grid live-traps (one trap every 12.5 m: Ugglan 2, Grahnab, Hillerstorp, Sweden). The bait consisted of a slice of apple and peanut butter. Hydrophobic cotton wool was also added to the traps to provide shelter from the cold and humidity.

The traps were checked every morning for 4 days, then remained opened for 3 days and finally were checked again once a day for 4 days. This was due to increased human presence during week-ends on the sites, exposing traps to theft or destruction. Captured bank voles and wood mice (*Apodemus sylvaticus*) were sexed and weighed (to the nearest 0.1 g). To be considered as adults, males needed

testes in scrotum while females needed perforated vagina, vaginal plug or distended abdomen linked to pregnancy. All rodents outside these criteria were considered as juveniles. For adult voles (N = 446) and adult mice (from site 2 only, N = 36) captured for the first time in a given 11-day session, a 2 µl blood sample was also taken with a micropipette after puncture of the saphenous vein. The presence of hantavirus IgG was tested with Ab-Dect Puumala rapid field test (Reagena, Toivala, Finland). Animals were individually marked with a numbered ear-tag (model 1005-1, National Band and Tag Co, Newport, KY) and subsequently released at the place of capture. This procedure was approved by the Nature Conservation Board of the Brussels Region.

Data analysis and statistics

For each vole captured in more than one trap in a given season, the maximal distance between the two most distant captures was calculated (as a straight line between two traps). This was corrected for body weight (by dividing by weight) and defined as index 1. For the animals captured in at least three traps in one capture season, their home range was estimated by the minimum convex polygon method (average number of captures = 4.25). For these animals, the maximal distance corrected for weight (index 1) and home range corrected for weight (defined as index 2) were also used. The seroprevalence was calculated for the tested voles only (which are probably the most active individuals with the highest infection probability) and for the whole population including the juveniles (which can be protected from hantavirus infection by maternal antibodies). The first method avoids the influence of the juveniles' abundance on the seroprevalence calculation. Data of human hantavirus infections in Belgium for the corresponding years as the rodent survey years were obtained from the Scientific Institute of Public Health (through the national reference laboratory and the sentinel laboratory network).

Next to confidence intervals and hypothesis tests for basic parameters, more elaborate and appropriate statistical models were constructed in order to investigate (1) which factors were statistically related to the probability for an individual rodent in the population to be positive and (2) which factors had a significant effect on the mean weight of voles in the population. More precisely, optimal univariate and multiple logistic regression models where fitted to investigate which of the factors site, season, year, sex, adult vole density, weight had a significant effect on the infection status. For part of the data also the maximal distance between two captures, the maximal area

between at least three captures (i.e. home range), index 1 and index 2 were available and were related to the infection status. The most complicated multiple logistic regression model, including all main effects and all interactions, was gradually reduced to the optimal submodel using a backward stepwise procedure. In a similar way, another multiple linear regression model was built to examine how the mean of the (continuous) response *log(weight)* (log transform was needed to satisfy normality assumptions) depended on all other above-mentioned factors as well as on the infection status.

Results

Populations and seroprevalence

The captured species were bank voles (N = 665, 75%) and wood mice (N = 220, 19%) but also shrews (*Crocidura russula* and *C. leucodon*, N = 31, 5%) and dormice (*Eliomys quercinus*, N = 2, 1%). Voles were captured during each of the four sessions (182 individuals in autumn 2003, 89 in spring 2004, 108 in autumn 2004 and 286 in spring 2005). No mice were captured in autumn 2004 (53, 24, 0 and 143 individuals during the four sessions). The proportion of juvenile voles did not differ between spring and autumn (spring: 32.5%, autumn: 37.4%, chi-square, N = 665, P = 0.43).

Univariate logistic regression analyses (with overdisperion accounting for unobserved heterogeneity) revealed that the seroprevalence is mainly influenced by the season. A higher seroprevalence was observed in the spring than in the autumn (Table 1). Also the vole density appeared to be an important factor with higher seroprevalence in high densities (see Fig. 1) and seroprevalence increased with weight (a proxy for age), as to be expected. Finally the univariate logistic analyses indicated that the capture site showed a borderline significant effect on the prevalence (p = 0.0572 between sites 1 and 2). Year and sex did not show significant effects.

The univariate logistic regression models were extended to a multiple regression model, allowing to study possible interaction effects. A stepwise model building procedure led to a final optimal model which improved the fit of all univariate models substantially. The final model included main effects site and season as well as an interaction effect of site and season. The interaction effect was highly significant (p-value 0.0017 for the interaction term season × site2 and 0.0276 for the interaction term season × site3, site1 served as reference), and consequently the main effects of site and season have no longer any direct interpretation (moreover they were not significant at level 0.05). The significant

interaction effect can be interpreted as follows: the odds for PUUV infection always increase in spring as compared to autumn, but this increase differs from site to site: the effect of the season is large for site 2, followed by site 3 and the smallest for site 1. A separate multiple logistic regression analysis was performed for those data for which index values were available. Again there were higher odds for Puumala virus infection in spring (independently of site) and the odds also increased with index 1 (maximal distance between two captures, corrected for body weight).

Finally, for wood mice, hantavirus status was only tested in site 2 in autumn 2003 and spring 2005. In autumn 2003, no positive wood mice were detected (N = 15), while adult voles similarly showed a very low seroprevalence (5%, N = 20). In spring 2005, however, male and female wood mice were seropositive for hantavirus. They showed a similar seroprevalence (62%, N = 21) than the voles (58%, N = 66), which was the highest one observed. A logistic regression on these four observations showed that seroprevalence is influenced by season (Estimate = 2.865, se = 0.903, P = 0.0015) but not by species (Estimate = -0.869, se = 1.719, P = 0.613).

Temporal and spatial analysis

The optimal multiple regression model (using stepwise model building) for the continuous outcome log(weight) included significant interaction effects season × site 2 (p-value = 0.0456) and season × year (p-value < 0.0001). The interaction effect season × site 3 was not significant (p-value = 0.2846) and site 1 served again as reference. As recommended in statistical literature, all main effects of season, site and year were retained in the model although the year main effect was not significant. Again, as interaction effects are present, main effects have no direct interpretation. Overall, mean weight (on log scale) was significantly higher in spring, but this seasonal effect differed from site to site (smallest for site 1 and largest for site 2), and differed with the years (smaller for autumn 2004 and spring 2005).

Secondly, 23 voles (and one wood mouse) were recaptured between two successive sessions, of which fourteen were tested in both sessions (being already adults in the first session). Twelve of them were negative during the first test and the two last ones were positive but did not show detectable level of IgG during the second test a few months later. So all the voles recaptured during two seasons were negative either for the first test or for the second test. On the whole, six (43%) of these 14 voles seroconverted between the two sessions; they all did so during winter.

In addition the adult vole densities (in average for the three sites and excluding juveniles) strikingly show similar temporal variations that the monthly average number of human infections recorded in Belgium (Fig. 2). If the vole juveniles are included, the temporal variations still show similar results.

Discussion

This 2-year study showed that bank voles were common at all three suburban sites studied and that hantavirus was detected in voles at least in one site during each of the four capture seasons. Voles showed large population variations while hantavirus seroprevalence also showed fluctuations. Voles almost disappeared locally but the virus came back with the new population the following year. Changes in vole demography were synchronous with those observed in wood mice. This result is consistent with both species experiencing the same environmental constraints as food limitation or predation risks.

This work determined four factors that had a significant impact on the seroprevalence of the studied populations. The main factor influencing the prevalence was the season with spring being a period with higher seroprevalence than autumn. This is probably linked to the presence of old overwintering voles in spring. Indeed tested voles were heavier (consequently older, as weight seems a good proxy for age) in spring than in autumn. There are two non-exclusive explanations to this observation. Firstly the winter weather can increase survival of the virus outside the host, which would thus raise indirect transmission from the environment, as already observed in cold winters in Belgium (Linard et al. 2007). Secondly voles living inside mixed wintering groups have a higher probability of encountering an infected vole (Escutenaire et al. 2002). Indeed the few seroconversions observed in this study all occurred during winter. The population structure in spring is thus a cohort of animals with a high likelihood of infection. The low seroprevalence in autumn could have been due to the summer reproduction leading to a large cohort of young animals with low seroprevalence due to maternal immunity. However the observed proportion of juveniles was not different between both seasons (spring: 32.5%, autumn: 37.4%).

The second significant factor for seroprevalence was vole density, as previously thought (Heroldová et al. 2010, Heyman et al. 2001). This is linked to the increased probability of rodent direct encounters and the larger presence of the virus in the environment at high density, two important direct and

indirect sources of virus transmission between rodents. Since the virus survival outside a host is limited to a few weeks (Kallio et al. 2006), the reoccurrence of hantavirus after local vole disappearance is probably due to the immigration of infected animals rather than the survival of the shed virus in the environment.

The third factor showing a significant influence on seroprevalence was vole weight. As seen in other hantavirus hosts, heavier (thus probably older) rodents have a higher probability to be hantavirus positive, given their longer period to have been in contact with the virus (Escutenaire et al. 2002, Heroldová et al. 2010). Weight is in turn depending on season, logically with bigger (older) animals in spring, but a deeper analysis showed that this seasonal variation in weight was dependent on site and on year: in the suboptimal site 2, weight difference were larger between spring and autumn while the difference was smaller in site 1. The influence of year is consistent with the multi-annual population dynamics (Tkadlec & Zejda 1998) and the analysis would benefit from data on longer periods.

The fourth factor that influenced the seroprevalence was the capture site. As, even the two sites that were very close to each other showed different seroprevalence dynamics, ecologic differences between the sites probably constitute a good explanation, as already observed (Escutenaire et al. 2002, Olsson et al. 2005). Site 2 was a fragmented suboptimal habitat, surrounded by low quality environment for rodents (no covering vegetation and large roads), unlike the two other sites (surrounded by forest). It is possible that the relative dryness of site 2 induced a low survival of hantavirus (Kallio et al. 2006). Moreover, as food did not seem a limiting factor and hantavirus infection does not wipe out rodents, different local human pressures can be another determining factor (such as destruction of burrow entrances or foraging runways). Finally the differences between the sites have certainly an influence on predator risks and emigration (Korpimäki et al. 2005), which have a drastic impact on population density and thus PUUV prevalence (Deter et al. 2008).

The discrepancy between the sites was further confirmed with the interaction between sites and season. A differential impact of season on the seroprevalence was indeed observed in the three sites. Season's effect was important for the suboptimal site 2, average for the undisturbed remote forest (site 3) and small for the travelled forest (site 1). Indeed in site 2, positive voles were found almost exclusively in spring. In site 3, positive voles were found during both season but with a higher seroprevalence in spring. In site 1, seroprevalence was quite stable through seasons. Environmental factors are known to have an impact on seroprevalence (Escutenaire et al. 2002; Heyman et al. 2009)

and the present analysis shed some light on the high variation between the sites close to each other. Interestingly the effect of site on seasonal weight shows a similar structure than that of seroprevalence described above (site 2 > site 3 > site 1).

The four significant factors for vole seroprevalence (season, vole density, vole weight and site) probably influence the human infection dynamics. Firstly, season is indeed important for human risk. In spring old rodents are looking for new resources to maximize their reproduction (Gliwicz 1990), i.e., they migrate or, at least, enlarge their home range. Breeding voles also increase their antagonistic behavior, which is linked to hantavirus transmission, and, at least in males, the marking of their home range (Escutenaire et al 2002). These behaviors probably expand the areas potentially contaminated by the virus and are consistent with higher numbers of human infections in spring or summer, period with increased outdoor activities in humans (Mustonen et al. 1998). Secondly, vole density is also important for human infection risk. Indeed the present study shows a similar profile between the average vole densities observed and the number of recorded human infections in Belgium (Fig. 2). No NE case was reported at close vicinity of the studied areas (but site 3 was not close to settlements). Hence it was not possible to directly link the observations of this study with a medical analysis. A further study targeting zones with human cases might be a solution to this, even if humans can be contaminated away from their home. In Sweden vole density is the main factor promoting NE cases (Palo 2009). As vole densities increase when seed production is high (mast year, Tersago et al. 2009), it would be possible to predict high-risk years by monitoring seed production. Another predictive possibility would be linked to the analysis of temperature and precipitation (Haredasht et al. 2011). The NE outbreaks are described as synchronized over a large area (e.g. Belgium and Germany, Heyman et al. 2007). This is linked to the comparable climatic conditions leading to synchronized mast years. However the season with most human cases may differ inside these areas. Therefore it is essential to understand the local conditions that could influence the virus transmission to humans (Schwarz et al. 2009). This certainly includes local vole population composition, which can be determined by weight, depending on the season and the local habitat conditions, as shown in this study (factors 3 and 4).

In the present study, no seroprevalence differences between males and females were detected (the juveniles were excluded from the analysis). It is not clear at what point sex is important in regard to PUUV infection as it can have a clear influence (Deter et al. 2008) or not (Escutenaire et al. 2000). An explanation for the absence of sexual difference would be that wounds, which are determining in

PUUV infection (Escutenaire et al. 2002), would be in excess at high density in sexually active territorial females. This would suggest that, under certain conditions, hantavirus can affect all adults in a given population at a similar rate. Usually male bank voles show home range larger than those of females (Bujalska & Grüm 1989). As home range size is indirectly related to the probability of being infected by hantavirus, males are typically considered as more at risk of being PUUV positive (Deter et al. 2008). This study indeed showed that voles with a larger distance between their two most distant captures showed higher probabilities to be PUUV seropositive.

It was also shown that, at least at high hantavirus seroprevalence in voles, wood mice can show peaks of hantavirus seroprevalence. Previous work reported very low PUUV seroprevalence in wood mice (Klingström et al. 2002) while the current study recorded a high seroprevalence in wood mice and voles (about 60%). As wood mice normally do not carry hantavirus, the serotype detected is possibly PUUV, given the common occurrence of this serotype when wood mice are infected. This suggests that PUUV can spill-over in wood mice at least at high bank vole seroprevalence. Such spill-overs are important for hantavirus evolution in new hosts (Schlegel et al. 2009).

Finally, in this work the inter-seasonal recapture rate was higher for negative animals in comparison to that of positive animals. This could be related to their younger age. However a detrimental influence of the infection by PUUV in voles cannot be eliminated, as shown by Kallio et al. (2007). As the production of antibodies has an energetic cost to the host, one could expect hantavirus infection to affect the host ability to reproduce or at least to increase the probability to concurrent infections. A few recaptured voles were positive in the first session but did not show a detectable level of antibodies against PUUV during the second session a few months later. This shows that positive voles can survive at long term. It also confirms that under natural conditions voles in chronic phase of the PUUV infection can stop to produce PUUV antibodies, confirming that they do not shed the virus at this stage (Bernshtein et al. 1999). However no vole that had remained positive between two sessions was recaptured. This suggests that PUUV infection could have a (long-term) survival cost in voles.

In conclusion, this study followed the temporal and spatial dynamics of small rodent populations infected by PUUV at close range of large human presence. It showed that season, population density, vole weight and site features were the key factors influencing seroprevalence. None of these factors alone was sufficient to explain the seroprevalence variations, demonstrating the complexity of the interactions. Furthermore this work showed that the vole local density was following a similar profile

than the numbers of NE cases in humans, which are partly influenced by season but probably also by local site variations. Vole sex had no significant influence in the system studied while home range size mattered. These results shed some light on a rarely studied type of rodent populations and on the factors useful for human risk evaluation.

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Tables

Table 1. Hantavirus seroprevalence (%) for vole weight classes in spring and autumn

	Spring		Autumn		
Weight class	Males	Females	Males	Females	Total
12.6- -16.5	57.1 (7)	33.3 (12)	0.0 (18)	7.7 (13)	26.0 (50)
16.6- -20.5	34.9 (43)	44.1 (34)	4.5 (44)	9.1 (33)	33.1 (154)
20.6- -24.0	42.2 (45)	32.1 (28)	0.0 (17)	11.1 (18)	31.5 (108)
24.1- -33.0	28.8 (52)	42.9 (35)	30.0 (10)	10.0 (10)	32.7 (107)
Total	36.1 (147)	39.4 (109)	5.6 (89)	7.7 (74)	25.8 (419)

The numbers of weighed bank voles are indicated in brackets. No weight or seroprevalence difference was detected between tested males and females. Seroprevalence (and weight) were higher in spring than in autumn. On the whole, positive voles were heavier than negative ones (see Table 2 and statistics in the main text).

Figure legends

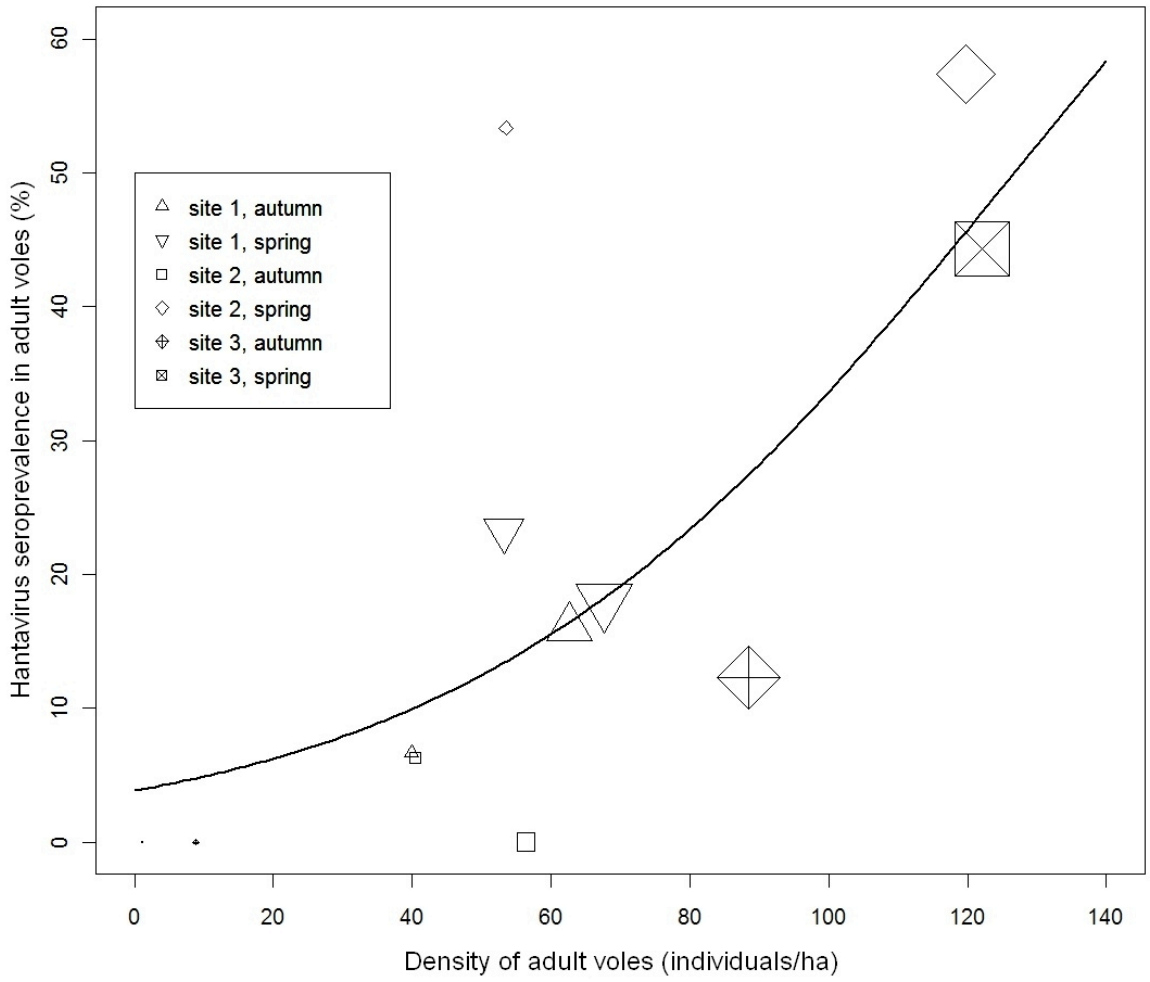

Fig. 1. Plot of seroprevalence data overlaid with fitted curve based on univariate logistic regression model with a linear effect of weight (linear on the logit scale, nonlinear on the prevalence scale). The symbols have size proportional to the number of observations for that particular combination of site and session (larger symbols have more impact on the model fit). One of the observations for site 3 in spring is barely visible (in the lower left corner) because it is based on one vole only.

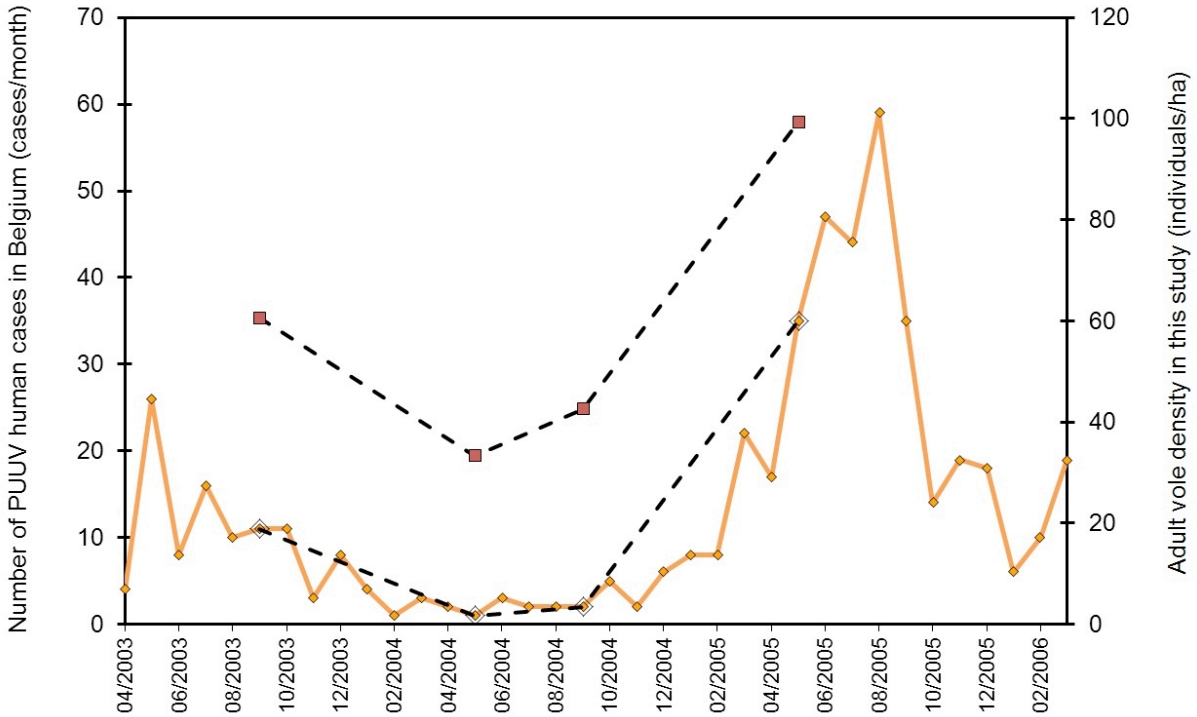

Fig. 2. Comparison of the monthly number of human hantavirus infections in Belgium (diamonds) and adult vole density from this study (excluding juveniles, squares). The changes in vole density are indicated by a dashed line. These changes are similar for the number of human infections between the same dates. The human data come from the Scientific Institute of Public Health, they were gathered by the sentinel laboratory network (www.iph.fgov.be/epidemio/labo) and the reference laboratory.