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Title — Schmallenberg virus, cyclical reemergence in the core region: a seroepidemiologic study in wild cervids, Belgium, 2012-2017

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Accepted Article

Abstract

Schmallenberg virus emerged in 2011 in Europe. The epicenter of primordial spreading was the region straddling Germany, the Netherlands and Belgium. One of the key questions is whether the newcomer would establish a lasting presence on the continent. The apparent seroprevalence in southern Belgium wild deer populations was followed for 6 years. Two years of intense circulation were revealed, 2012 and 2016, characterized by a peak seroprevalence in the two studied populations (*Capreolus capreolus* and *Cervus elaphus*). Between the peak years and after 2016, apparent seroprevalences declined rapidly among adults and became nil among juveniles. The general pattern of apparent seroprevalence evolution observed is consistent with a cyclic circulation of Schmallenberg virus, similar to what is observed for other Orthobunyaviruses in endemic areas. These data also suggest that wild cervids play no central role in the circulation dynamics of the virus.

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Introduction

Schmallenberg virus (SBV) is a tri-segmented negative-sense RNA virus that was discovered in 2011 in Germany and the Netherlands after an increasing number of veterinarian practitioners reported a syndrome of fever, watery diarrhea and drop in milk production in cattle herds that was not solved by an extensive etiologic investigation (Hoffmann *et al.,* 2012*;* Muskens *et al.,* 2012*).* The SBV is one of the 88 members of the *Orthobunyavirus* genus that belongs to the *Peribunyaviridae* family. It infects ruminant species and is transmitted by midges from the *Culicoides* genus (Diptera: *Ceratopogonidae*). The most efficient vectors belong to the Obsoletus complex (C. *obsoletus*, C. *chiopterus*, C. *scoticus*) (De Regge *et al.,* 2014; Rasmusse *et al*., 2012*)*. At the farm level, the major economic impact of the infection results from infection of fetuses (Häsler *et al.,* 2015; Saegerman *et al*., 2014; Waret-Szuka *et al.*, 2017), in which the virus causes either mortality or very significant deformities of the appendicular system, themselves caused by severe neuronal losses in the brain and spinal cord (Bayrou *et al.,* 2014).

One of the most spectacular aspect of the newcomer biology was its remarkable speed of spreading across Europe (Rossi *et al.*, 2017). Retrospective studies in *Culicoides* populations across France showed that, by the time of the virus discovery by the Friedrich-Loeffler-Institute (Germany) in automn 2011, SBV had already invade the entire North-East quarter of France (Ségard *et al*., 2018). After the first season of circulation, the virus geographic distribution area covered Belgium (Méroc *et al*., 2013), the Netherlands (Elbers *et al., 2013*), the northern half of France (Dominguez *et al.*, 2012), most of the German territory (Conraths *et al.*, 2013) and the south of England (McGowan *et al*., 2018). Compared to the blue tongue virus serotype 8 (BTV-8) that emerged in the same area in 2006, SBV spreading was evaluated to be 20 times more "efficient" in terms of cumulative cases and 2 times in terms of distances covered (Gubbins *et al*., 2014).

Belgium was one of the core countries during the primary epidemic. In spring 2012, the seroprevalence in cows from Wallonia (southern half of Belgium) reached 90,8% (Garigliany *et al*., 2012) and the herd-prevalence in the whole country was 99,8% in cattle (Méroc *et al*., 2013) and 98,0% in sheep (Méroc *et al*., 2014). All across Europe, wild ruminants that have been in contact with the virus developed a detectable humoral response (García-Bocanegra *et al*., 2017; Larska *et al*., 2014; Linden *et al*., 2012; Mouchantat *et al*., 2015; Rossi *et al.,* 2017). Beside the

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detection of SBV-specific antibodies, no evidence of other signs of the disease was reported, especially no abortion.

At the end of 2011, seroprevalences in wild ruminants populations in Wallonia were 45.9% in roe deer (*Capreolus capreolus*) and 40.5% in red deer (*Cervus elaphus*) (Linden *et al., 2012*). We herein followed the dynamic of seroprevalence to SBV over 6 consecutive years in the same species and across the same region. According to our best knowledge, this is the first study so far offering a 6-year global view of the virus fate after the primary outbreak in a region (Wallonia, southern Belgium) among the first affected after the SBV emergence.

Materials and methods

Ethical statement — All the samples used in this study were collected post-mortem. As a consequence, no animal being specifically hunted for this study, no ethical approval was required.

Serum samples — The study was conducted in Wallonia (southern Belgium, ~16.900 km^2). Through a targeted surveillance program 2012-2017, wild cervids were sampled by the Surveillance Network of Wildlife Disease (Linden et al., 2011) during 6 hunting seasons (October to December). During that period, the deer population was decreasing in Wallonia. Based on the hunting bags, on average over the study period, the roe deer population was estimated around 30,000 animals and the red deer population around 9,000 animals.

Samples were carried out in the 5 provinces of Wallonia, taking into account the wild cervids populations living in those provinces. Roe deer is widespread over all of Southern Belgium and red deer mainly lives in the large forested areas of the provinces of Luxembourg and Liege (Casaer and Licoppe, 2010) in the belgian part of the Ardennes forest (Fig. 1).

A two-stage cluster sampling was carried out during each hunting season : firstly, some hunting areas were randomly chosen in several municipalities by province and, secondly, some animals were randomly sampled in each hunting area. Sampling was conducted each year in the same provinces (Table 2).

All animals were necropsied in the hunting grounds, within 2 to 3 hours after shot. Individual post mortem examination included determination of sex, age and body weight. The age was determined according teeth eruption pattern (Azorit *et al.*, 2002). After examination of the intact whole body, abdominal, thoracic and naso-buccal cavities and corresponding organs were systematically checked. Afterwards, blood was immediately collected in dry tubes exclusively by venipuncture

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from major vessels (in priority femoral vein, posterior vena cava or jugular vein) or cardiac puncture. Samples were transported to the lab within 12 hours and after centrifugation, sera were stored at -20 °C.

Blood was collected in both species (from 225 to more than 380 samples per season) allowing the theoretical detection of a seroprevalence of 18 % and 50 % with an accepted error of 5 % and a confidence level of 95 % (Thrusfield *et al.*, 2001).

A set of descriptive data was systematically aggregated with each serum : species, gender, age, body weight, date and location of sampling.

Detection of antibodies — All samples were analyzed using the commercially available ID Screen® *Schmallenberg virus Competition Multi-species* ELISA assay (ID.Vet, Grabel, France). Following the manufacturer instructions, the analysis can lead to 3 different outcomes: positive, doubtful or negative. All sera were tested twice, those returning either 2 positive or 1 positive and 1 doubtful results were considered as positive. To assess the relative sensitivity (Se) and specificity (Sp) of the said ELISA assay, sero-neutralisation assays (SN) were carried out in parallel on a random subset of samples taken from both species in years 2012 to 2014. After thawing, these sera were incubated at 56°C during 30 min for inactivating the complement. SN was performed after 2 h incubation of a series of consecutive 2-fold dilutions for each serum sample with 100–200 tissue culture infectious dose fifty of SBV (isolate SBV-BH80/11–4, kindly provided by University of Namur). Virus back titration was performed onto BHK cell monolayers using standard procedures. Titers ≥ 8 were considered positive.

Statistical analysis — Descriptive statistics were used to calculate the frequency of seropositive animals by species and year. A logistic regression was applied to the whole dataset and, separately, to each species (*Capreolus capreolus – Cervus elaphus*). The following factors were used to explain the positive ELISA results: “year of sampling”, “month of sampling”, “municipality (location)”, “East-West position”, “North-South position”, “species”, “gender” and “age” (adult or juvenile). The statistical differences between years and species were evaluated using a *post hoc* test. All statistical analysis were performed with SAS.

Results

The species, age, time and location distributions of animals from which serum samples were drawn are detailed in Table 1 and Fig. 1. In this study, 2258 sera were analyzed : 1140 from roe

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deer and 1118 from red deer. Most wild cervids living in southern Belgium are found in the Ardennes, wich explains the apparently heterogeneous geographical distribution seen in Fig. 1.
To assess the relative sensitivity and specificity of the commercial ELISA used in this study, 622 sera were randomly drawn from the 2012, 2013 and 2014 cohorts for simultaneous testing by SN, the latter being taken as the gold standard. The overall relative Se was 70% (95% CI: 66% - 77%) and Sp was 93% (95% CI: 90% - 96%). For unknown reasons, the relative Se dropped dramatically to 30% when examining the subcohort of red deer sampled in 2012 only. These sera were retested with different kits/operators without improving the detection rate of positive samples. Based on this abnormal low level of detection compared to all other subcohorts, we decided (Hoffmann *et al.,* 2012) to report overall Se and Sp calculated from the 5 other subcohorts, (Muskens *et al.,* 2012) to use SN-calculated seroprevalence in the said subcohort and (Rasmussen *et al*., 2012) to exclude red deer sampled in 2012 from the statistical analysis. To ensure that no other drop in the sensitivity of the ELISA occured in the years 2015 to 2017 for the red deer population, we performed SN assay on 20% of the negative sample of those three years. The results confirmed a sensitivity of the ELISA close to 70%. This extra set of SN allows us to confrim that the low seroprevalence detected in 2015 and 2017 was not the consequence of an ELISA dysfunction.
The logistic regression detected two factors that significantly influenced the probability to be positive : “year” and “age” ($p < 0.0001$). The apparent seroprevalence results suggest that only two years were compatible with an intense virus circulation : 2012 and 2016 (Fig. 2). Indeed, for both years, a far higher apparent seroprevalence than for other years of sampling was observed, whatever the species or the age category ($p < 0.01$). Among juveniles, the apparent seroprevalences in 2013, 2014, 2015 on the one hand and 2017 on the other were not significantly different from zero ($p > 0.05$). Simultaneously in the adult populations, a continuous decrease in apparent seroprevalences was noticed from 2012 to 2015 compared to 2012, then in 2017 compared to 2016 (with a nadir close to 10%). The rate of decrease tend to be faster among roe deer compared to red deer.

Discussion

The relative sensitivity and specificity of the ID Screen® *Schmallenberg virus Competition Multi-species* ELISA assay were 71% and 93%, respectively. At first sight, these results appear quite

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low in comparison to what is usually reported for domestic ruminant species, with more than 98% of concordance (van der Heijden *et al.,* 2013*;* Bréard *et al.,* 2013*;* Loeffen *et al.,* 2012). However, the said ELISA assay is widely used in wildlife with a sensitivity close to ours : ~70% (Rossi *et al.,* 2017*;* Laloy *et al.,* 2014*;* Molenaar *et al.,* 2015). Unexpectedly, we found that the sera from red deer collected in 2012 yielded dramatically lower seropositive counts by ELISA than by the SN assay run in parallel. In brief, the ELISA assay sensitivity was dramatically lower for the red deer/2012 subcohort than for any other subcohort, whatever the year or the species. A possible role of the operator or of the batch of the ELISA plates was ruled out leaving us with a set of unsolved questions. To avoid distortion of the data presented, we decided to discard the ELISA results from this subcohort and to replace them by the results yielded by the SN assay.
At the outset of the study, it was well-grounded to focus on wild ruminants since their susceptibility to SBV infection had been duly demonstrated previously (Linden *et al.,* 2012). In addition, these species display two advantages when the goal is to examine the biology of SBV. First these animals live 24 hours a day outdoors, which maximizes their exposure to infected vectors, thus the probability of seroconversion. This is not a small advantage since we know that even partial indoor management influences seroprevalence (Helmer *et al.,* 2013*;* (Veldhuis *et al.* 2013). Secondly, the hunting season in Belgium being limited to the months of October to December, the collection of samples systematically took place at the end of the *Culicoides* season, which also maximizes the exposure of the animals to infected vectors (Cuéllar *et al.* 2018). Besides, as we dealt with a population of wild animals subject to hunting, a limitation of the study comes from the difficulty of conducting a preestablished statistical sero-survey.

Cyclical extensive circulations — The results suggest an endemic installation of SBV with cyclical major reemergence, otherwise known as endemic pulsations (Thrusfield, 2018), which is highly reminiscent of its closest phylogenetic relatives Akabane, Aino and Peaton viruses' biology (Kato *et al.,* 2016). This cyclical profile fits with the "Susceptible-Infected-Recovered-Susceptible" (SIRS) epidemiological model. Transition from "recovered" to "susceptible" is expected to involve 3 main mechanisms : population renewal, vanishing immunity or viral mutations. Though the latter mechanism has been regularly observed with RNA viruses, it is unlikely it plays a role here because SBV displays a remarkable genetic stability over time which is a consequence of the bottleneck effect created by the permanent shift between mammalian and arthropods hosts (Hofmann *et al*., 2015; Wernike *et al.,* 2017). Acquired immunity against SBV

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has been demonstrated to last at least 4 years in sheep (Claine *et al.,* 2018) and 6 years in cattle (Wernike *et al.,* 2018) and could be lifelong. Even if the duration of immunity varies among animals, those estimations do not fit with a SIRS model where vanishing immunity is the main factor. Should the duration of acquired immunity in wild cervids mimicks that in domestic species, then the rate of population renewal appears to be the main, if not the sole driver of SBV infection's cyclicity. Here, however, we show that the level of anti-SBV antibodies decreases very rapidly, particularly in roe deer : from 44% in 2012 to 14% in 2013 and from 47% in 2016 to 17% in 2017. If the population rate of renewal was the only factor explaining this drop in seroprevalence, the calculated renewal rate of the roe deer population would exceed 60%, which is not consistent with the maximum rate (~40%) observed for this species (ONCFS, 2019). As a result, the study suggests that in roe deer population, the rapid collapse of humoral immunity also contributes to the rapid decline of the seroprevalence.

Since juveniles are born in spring before the *Culicoides* season, their seroprevalence assayed between October and December theoretically reflects the extent of virus circulation in their year of birth. In principle, it may be objected that the humoral immunity conferred on them by the ingestion of maternal colostrum should condemn their use as sentinels of the viral circulation during their year of birth. Maternal antibodies persist for 4 months in lambs (Claine *et al.* 2018) and 6 months in calves (Elbers *et al.* 2014). By extrapolating to deer and knowing that births take place between mid-May and mid-June, it follows that a significant proportion of juveniles should still have maternal antibodies between October and December. However, juveniles born in 2013 or 2017 have a seroprevalence that tends to zero whereas about 50% of the corresponding mothers must have produced colostrums rich in anti-SBV antibodies, a consequence of their infection during the previous year (Fig 2). It is concluded that (Hoffmann *et al*., 2012) passively colostrum-transmitted anti-SBV maternal immunity persists for a much shorter time in roe/red deer than in domestic ruminants and (Muskens *et al*., 2012) that SBV circulation decreases very rapidly after a peak year. A similar observation was made in the context of the BTV-4 circulation in red deer in Spain (Falconi *et al.*, 2012) were calves sampled during the hunting season were used as markers of the virus circulation over the *Culicoides* season of their year of birth.

Extensive circulation in 2012 — The results show that SBV circulated extensively among roe/red deer in 2012. Interestingly, infections were only occasionally recorded among domestic ruminants across southern Belgium over the same period (Bayrou *et al.,* 2013). As the majority of domestic

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ruminants had already massively seroconverted (>85%) before the 2012 culicoid season (Garigliany *et al.,* 2012; Méroc *et al*., 2013; Méroc *et al.,* 2014), not enough naive animals remained available to sustain a significant circulation of the virus in farms. Comparatively, far less wild ruminants (~40%) had been infected in 2011 (Linden *et al.,* 2012), which explains why the virus circulation was more extensive among them in 2012. In domestic cattle, however, it should be remembered that the magnitude of viral circulation was geographically heterogeneous in 2012. The circulation in cattle herds was much more intense in the Ardennes than anywhere else in Belgium : in calves aged 6 to 12 months, seroprevalence increased from 57% in autumn 2011 to 79% in 2012, while it decreased everywhere else (Méroc *et al.,* 2015). As shown in Fig. 1, wild cervid populations live mainly in the Ardennes massif. Thus, in both domestic and wild ruminants, the initial spread of the emerging virus was slower in the Ardennes biotope, which is characterized by a higher altitude than elsewhere in Belgium and is mainly composed of large forests and a mix of grasslands and wastelands. It is interesting to note that a comparable epidemic lag has been observed in the mountain ecosystem in France (Rossi *et al.,* 2017).

Extensive circulation in 2016 — A major SBV circulation was recorded in 2016 in both deer populations. This reemergence was also detected in domestic ruminants across all Europe, in Belgium (De Regge *et al*., 2014), Germany (Wernike *et al.,* 2017), France (Gache *et al.,* 2018), United Kingdom (Stokes *et al.,* 2018) and Poland (Larska, 2018). The decreased seroprevalence recorded in 2017 in this study suggests that another SIRS cycle is ongoing. During the 2011 first diffusion of the virus, Linden and colleagues (Linden *et al.,* 2012), studying the same species in the same area, noticed an increasing prevalence over the hunting season, suggesting ongoing infections occurred until mid-November. In 2016, this trend was not observed (« month » effect : $p > 0.05$), suggesting that the virus circulated earlier than in 2011.

Years with limited circulation — Between the two peak years, the apparent seroprevalences were very low especially in juveniles. Interpretation of the very low seropositivity is not straightforward, mainly because duration of the maternal immunity in roe/red deer is not known. Considering an annual rate of seroprevalence recession constant and equal to that measured between 2012 and 2013, the apparent seroprevalence measured at the roe deer population level (adults and juveniles) should have stabilized at around 2% in 2015. Since it is 10% in reality (Fig. 2), we deduce that the virus circulates at low level, probably locally.

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Wild ruminants as SBV reservoirs — At the beginning of the SBV outbreak the question of the possible implication of the deer as reservoir raised. At first sight, the apparent seroprevalence rates reported here suggest that wild cervids could serve as virus amplifiers or reservoirs. Nevertheless, our study was not designed to look after viremia levels that could be informative on that particular question.

Yet, it is noteworthy that the low levels of prevalence (around 10%) that precedes the 2016 reemergence were already achieved in 2014 for red deer and in 2013 for roe deer. Hence, if deer were a major reservoir for the virus, the 2016 circulation would have taken place earlier. It suggests a minor role of deer in the epidemiology of the SBV and, probably, the infection in wild deer follows the pace of the domestic outbreaks. The role of deer in the SBV biology depend on the efficiency of the SBV infection in those animals but also on the *Culicoides* feeding behaviour.

Roe vs red deer — Interestingly, in this study as in others (Linden *et al.,* 2012, Rossi *et al.* 2017; Vengušt *et al*., 2020), apparent seroprevalences tend to be close or slighly higher in roe than in red deer (even if not statistically significant in our study,). An opposite situation was found in the context of the BTV-8 epidemic that emerged in the same area in 2006 (Linden *et al.,* 2010). Roe deer were dramatically less infected by BTV-8 : seroprevalences were 2.8% and 52.3% in roe and red deer, respectively. At the time, one of the hypotheses to explain this huge difference was that *Culicoides* were much more likely to be attracted by red deer than by roe deer. This assumption implied that female culicoids detected their mammalian targets by sensing the volatile compounds emitted into the atmosphere, so that the size of the target played a role (Koch *et al.* 1979; Zimmer *et al.* 2015). Since both BTV-8 and SBV are transmitted by the same *Culicoides*-vectors, the results gathered here formally refute this assumption. The roe deer very low apparent seroprevalence to BTV-8 is therefore caused by another mechanism, yet to be discovered. It has to be noted that in Spain (Jiménez-Ruiz *et al.*, 2020), the red deer population showed a statistically higher seroprevalence in comparison to roe deer, respectively 31.6% versus 17.5%. Even if the difference is not comparable to what Linden and colleagues found for BTV-8, it raises the question of the factors which impact the seroprevalence. An important driver of the seroprevalence in cervid populations could be the contact with domestic ruminants (Fernández-Aguilar *et al*., 2014). Moreover, isolated ecosystems can impact the *Culicoides* ecology and thus the

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seroprevalence in ruminants populations. An illustration of this is the seropositivity detected in the red deer from zoo in Spain which was as high as 58,3% (Caballero-Gomez *et al.*, 2021).

The present multi-year follow up of apparent seroprevalence to SBV in wild red and roe deer living in southern Belgium suggests an endemic installation of SBV with endemic pulsations, which is highly reminiscent of the biology of its closest phylogenetic relatives in the *Bunyaviridae* family. This cyclical profile fits with the "Susceptible-Infected-Recovered-Susceptible" (SIRS) epidemiological model in which transition from "recovered" to "susceptible" is likely to result from a combination of 2 factors: population renewal and vanishing immunity.

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Conflict of interest

This study was conducted and manuscript prepared without any conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author, [DD], upon reasonable request.

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Figure Legends

Fig. 1 — Geographical distribution of wild cervids sera recruited for the study. Circles represent samples collected by the Surveillance Network of Wildlife Diseases of the University of Liège (Belgium) for (a) roe deer (*Capreolus capreolus*) population and (b) red deer (*Cervus elaphus*) population. Each circle is centered on the municipality where the samples were harvested. The circle area is proportional to the number of samples collected in that particular municipality. The map was edited in 2017 by the Walloon Institute for Evaluation, Prospective and Statistics (IWEPS) and show the use of lands in the provinces of Wallonia (Belgium).

Fig. 2 — Follow-up of apparent seroprevalence (with 95% confidence intervals) to Schmallenberg virus among (a) roe deer (*Capreolus capreolus*) and (b) red deer (*Cervus elaphus*) populations over the five years that followed the seeding and initial spreading in 2011. All data were obtained from the multispecies competition ELISA, except those drawn from red deer in 2012 (asterisk, from SN assay). See text for key. Statistical significance is indicated in tables under the bar graph of each species (p : p-value <0.05; / : no statistical test due to the methodology discrepancy).

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Table 1 — Species, age and time distribution of wild cervids sera recruited for the study

		2012	2013	2014	2015	2016	2017	TOTAL
Roe deer	Adults	169	186	121	147	125	76	**824**
	Juveniles	70	73	49	43	48	33	**316**
	TOTAL	**239**	**259**	**170**	**190**	**173**	**109**	**1140**
Red deer	Adults	106	140	118	99	103	65	**631**
	Juveniles	83	105	96	72	80	51	**487**
	TOTAL	189	245	214	171	183	116	**1118**
TOTAL		**428**	**504**	**384**	**361**	**356**	**225**	**2258**

Table 2 — Annual sampling (October to December) carried out in roe and red deer in the 5 provinces of Wallonia *(in brackets : number of municipalities where the sampling took place)*

		2012	2013	2014	2015	2016	2017	TOTAL
Roe deer	Walloon Brabant	4 *(1)*	4 *(2)*	7 *(2)*	9 *(1)*	11 *(1)*	0	**35 *(3)***
	Hainaut	12 *(3)*	9 *(2)*	10 *(2)*	11 *(2)*	6 *(1)*	7 *(4)*	**55 *(7)***
	Liege	98 *(15)*	78 *(15)*	45 *(9)*	54 *(14)*	41 *(7)*	29 *(6)*	**345 *(23)***
	Luxembourg	90 *(18)*	114 *(23)*	77 *(20)*	83 *(20)*	85 *(21)*	52 *(20)*	**501 *(37)***
	Namur	35 *(9)*	54 *(14)*	31 *(9)*	33 *(11)*	30 *(10)*	21 *(7)*	**204 *(21)***
	TOTAL	**239 *(46)***	**259 *(56)***	**170 *(42)***	**190 *(48)***	**173 *(40)***	**109 *(37)***	**1140 *(91)***
Red deer	Walloon Brabant	0	0	0	0	0	0	**0**
	Hainaut	0	0	0	0	0	0	**0**
	Liege	72 *(12)*	66 *(12)*	56 *(6)*	48 *(7)*	49 *(5)*	56 *(9)*	**347 *(17)***
	Luxembourg	78 *(16)*	144 *(19)*	107 *(19)*	89 *(21)*	84 *(16)*	31 *(12)*	**533 *(28)***
	Namur	39 *(7)*	35 *(8)*	51 *(5)*	34 *(9)*	50 *(8)*	29 *(6)*	**238 *(13)***
	TOTAL	**189 *(35)***	**245 *(39)***	**214 *(30)***	**171 *(37)***	**183 *(29)***	**116 *(27)***	**1118 *(58)***
TOTAL		**428**	**504**	**384**	**361**	**356**	**225**	**2258**

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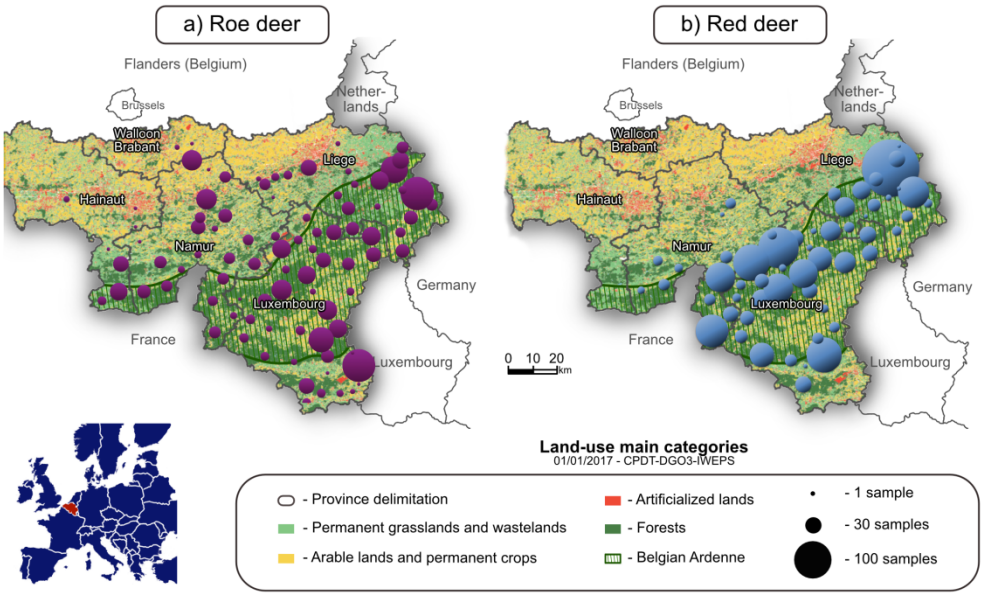

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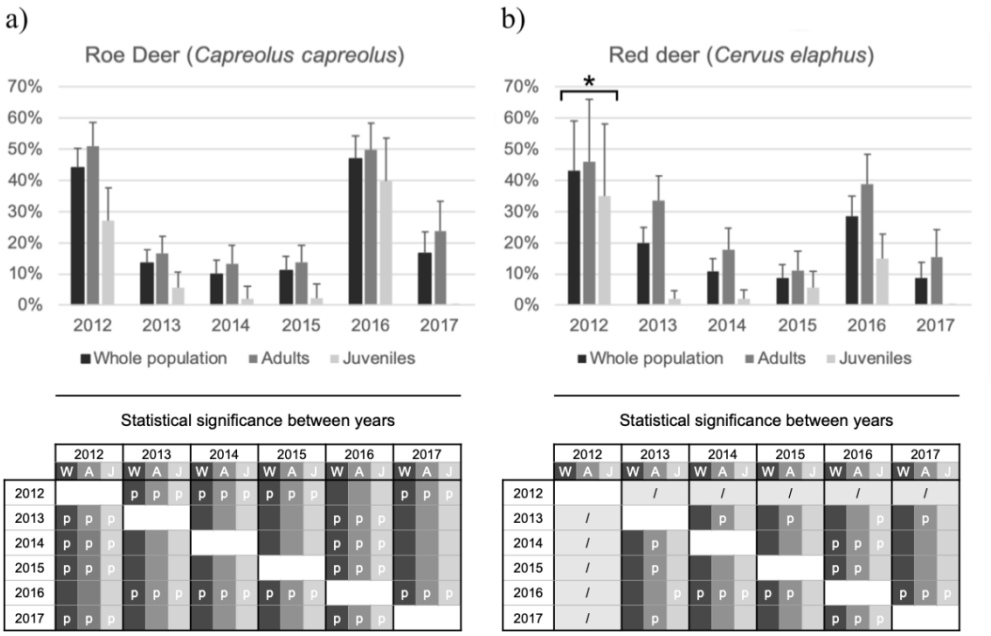

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