Likely circulation of a coronavirus in roe deer (*Capreolus capreolus*) populations suggested by SARS-CoV-2 serological investigation in France

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Abstract

The risk of viral transmissions from domestic and wild animals to humans is of high concern for human health. Humans can also transmit viral infections back to domestic and wild animals, which can then act as reservoir for the maintenance of viruses, with the risk of epidemic diseases re-emergence. The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), causing the COVID-19, likely originated from wildlife and has already been evidenced to be transmitted from humans to captive, domestic and wild animals. In particular, white-tailed deer (*Odocoileus virginianus*) show high-prevalence of SARS-CoV-2 following human contamination, and recent data suggest that white-tailed deer could act as an emerging virus reservoir. Here, we investigated whether SARS-CoV-2 could also have emerged in several longitudinally-monitored populations of European roe deer (*Capreolus capreolus*) in direct contact with humans in France. We performed indirect tests (serological ELISAs and seroneutralization) on serum collected pre- and post-emergence of the virus in human populations. We also performed direct tests (nasal swabs followed by RT-PCR) to detect the presence of SARS-CoV-2 RNA in those populations in 2022. We also investigated the exposure and prevalence of the virus in three other cervid species in France. ELISA tests were positive for 2.20 % of the sera tested, both pre- and post-emergence of the virus, but all positive samples were negative with seroneutralization. Direct PCR testing showed no positive results in 2022. Thus, although seroprevalence increased after the COVID-19 emergence (2022) in one out of three roe deer population, our results suggest that SARS-CoV-2 has not emerged in those populations, and that ELISA cross-reaction with another circulating virus, possibly an unidentified bovine coronavirus, is possible.

Keywords: COVID-19, wild ungulates, cervids, spill-over, spill-back, zoonoses

1. Introduction

The potential transmission of viral infections inducing an epidemic from livestock or wildlife to humans is of high concern for human health worldwide, as vertebrates constitute the main source of human emerging diseases (Jones et al. 2008). Conversely, human pathogens can be transmitted to domestic or wild animal species, potentially inducing new epidemics in these species (*e.g.* human coronavirus OC43 outbreak in wild chimpanzees; Patrono et al. 2018). Thus, there is a need to identify pathogens features (*e.g.* mutation rate, transmission route) and ecological contexts (*e.g.* species interactions, habitat use) that promote such events.

Coronaviruses are hosted by many mammal species and can be transmitted from one species to another, including humans (Monchatre-Leroy et al. 2017, Patrono et al. 2018, Alluwaimi et al. 2020). The COVID-19 pandemic, due to the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), most likely originated from wildlife (*e.g.* Lytras et al. 2021, Domingo 2022). Since the beginning of the COVID-19 pandemic, transmission events have been documented from humans back to domestic animals, for example farmed minks (*Neovison vison*; Oreshkova et al. 2020, Oude Munnink et al. 2021), domestic cats (*Felis catus*) and dogs (*Canis lupus familiaris*; Hobbs and Reid 2021, Krafft et al. 2021) or wild felids kept in zoos (McAloose et al. 2020). These first observed transmission events occurred in domestic, captive or intensive farming contexts and could be explained by elevated rates of direct contacts between humans and animals in such specific contexts. However, multiple events of transmission of SARS-CoV-2 from human to free-living white-tailed deer (*Odocoileus virginianus*) have also been documented in North America. These events were followed by transmissions within local white-tailed deer population, resulting in populations displaying high prevalence levels which were up to 40% by seroprevalence, and 82.5% and 70% in retropharyngeal lymph node and nasal swabs tested by RT-PCR,

respectively (Chandler et al. 2021, Hale et al. 2022, Kuchipudi et al. 2022, Feng et al. 2023). Eventually, three cases of transmission from the newly infected white-tailed deer populations to humans were detected, suggesting that the white-tailed deer may become an emerging virus reservoir in North America (Feng et al. 2023). Similar contamination pathways can be expected in Europe, as several closely-related species of cervids are highly abundant and can be exposed to viral transmission. For instance, first cases of seropositivity have been recently observed in fallow deer (*Dama dama*) and red deer (*Cervus elaphus*) in free-living suburban populations in Spain, suggesting that the host tropism of SARS-CoV-2 may change as new variants emerge (Encinas et al. 2023).

Among European cervid species, the roe deer (*Capreolus capreolus*) is particularly interesting because the species occupies a large variety of habitat, including agricultural areas with proximity to domestic species and human activities (Hewison et al. 2001), making roe deer potentially exposed to human-borne viruses. The species may also display a gregarious behaviour in winter in open landscapes (Gerard et al. 1992), favouring disease transmission. Furthermore, the hunting regulations and the absence of large predator during the last 50 years resulted in high abundance of roe deer all over Europe. Between 1984 and 2005, the number of roe deer in Europe was estimated to increase from 6.2 to 9.5 million and hunting grew from 1.7 to 2.7 million of individuals yearly (Burbaite and Csanyi 2009). In France, the roe deer is widely hunted with about 600,000 individuals officially hunted in 2022, *i.e.* ten times the number hunted 50 years ago (OFB, Réseau Ongulés sauvages 2022), thus increasing the rate of potential contact with humans during hunting or capture operations, one of the potential routes of transmission of the virus from humans to cervids (Fernández-Bastit et al. 2023).

Benefiting from longitudinal studies of four free-living (including three enclosed) and one captive populations of roe deer in France, we investigated whether SARS-CoV-2 had recently emerged in these populations subject to hunting or direct handling as part of capture-

mark-recapture programs. Because of annual captures, roe deer from these populations are particularly exposed to human viruses' transmission. Moreover, the continuous follow-up of the same individuals from 2010 to 2022 was an opportunity to detect virus arrival in populations and to explore associated individual factors, namely age and sex. Indeed, older individuals and males are expected to have higher seroprevalence as observed in white-tailed deer, likely driven by sex- and age-specific behaviours during reproductive periods (Hale et al. 2022, Caserta et al. 2023). The virus was searched for by means of either direct PCR testing (on individuals captured in 2022) or indirect serological testing to assess exposure before and after SARS-CoV-2 emergence in human in France in 2020. We further investigated exposure and presence of the virus in other cervid samples collected in 2022 during capture or hunting operations: red deer, fallow deer and sika deer (*Cervus nippon*).

2. Material and methods

2.1. Sampling

Sera sampling is summarized in Figure 1 and Table 1.

2.1.1. Roe deer populations

Roe deer sera were sampled from five populations: Trois-Fontaines (TF), Chizé (CH), Aurignac (AU), Gardouch (GA) and Domain of Praillebard (DP). Among these populations, three are hunted wild populations: TF, CH and AU (Fig. 1, red squares). TF is a 1,360 ha fenced forest in north-eastern France (48°43'N, 4°55'E) hosting an enclosed wild roe deer population; CH is a 2,614 ha fenced Integral Biological Reserve (unmanaged forest) since 2006 located in western France (46°05'N, 0°25'W) hosting also a closed wild roe deer population; AU is an open study area covering about 7,500 ha in south-western France (43°16'N, 0°53'E) including forests and agricultural landscapes with free-ranging roe deer.

Captive roe deer were sampled for their sera in GA (Fig. 1, orange triangle), an experimental station in southern France (43°22’, 1°40’E) hosting captive roe deer in small 0.5 ha enclosures or semi-captive roe deer in a large enclosure composed of 9 ha of forest and 5 ha of meadows.

Roe deer sera were also sampled at the DP (Fig. 1, yellow diamond), a private fenced area of 148 ha in eastern France (45°57’N, 4°55’E) hosting a small roe deer population introduced from TF and CH in 2019-2020.

2.1.2. Sera collection

Sera were collected in these populations during 2,134 capture events, involving 1,289 different individuals. Each individual was sampled one to nine times during the study period (2010-2022), with 874 individuals sampled only once. All samples were collected each year in winter between December (year *t-1)* and March (year *t*), except at GA where sampling occurred until May.

2.1.3. Nasal swabs collection

For direct virus detection, nasal swabs were collected in 2022 during the roe deer capture-mark-recapture programs from TF (n = 49), CH (n = 95) and AU (n = 61).

2.1.4. Additional sampling on other cervid species in other locations

Additionally, we sampled cervids in other locations: the Vercors Vie Sauvage (VVS) private reserve located in eastern France (44°57'N, 5°14'E; Fig. 1, green star) and three departments of central France (Allier, Cher and Loire; Fig. 1, blue areas).

We sampled 30 sera from other cervids captured in 2022 in the VVS, including 16 European fallow deer, five sika deer and nine red deer (Table 1). In the three central France departments, nasal swabs were also collected on hunted roe deer (Allier: n = 3, Cher: n = 29, and Loire: n = 4), and on red deer in one department (Cher: n = 3) (Fig. 1, blue areas).

We defined two periods, either before COVID-19 emergence in France (pre-COVID-19 emergence: 2010-2020, n = 1,752, 'pre-Cov' hereafter) or after COVID-19 emergence (post-COVID-19 emergence: 2021-2022, n = 382, 'post-Cov' hereafter).

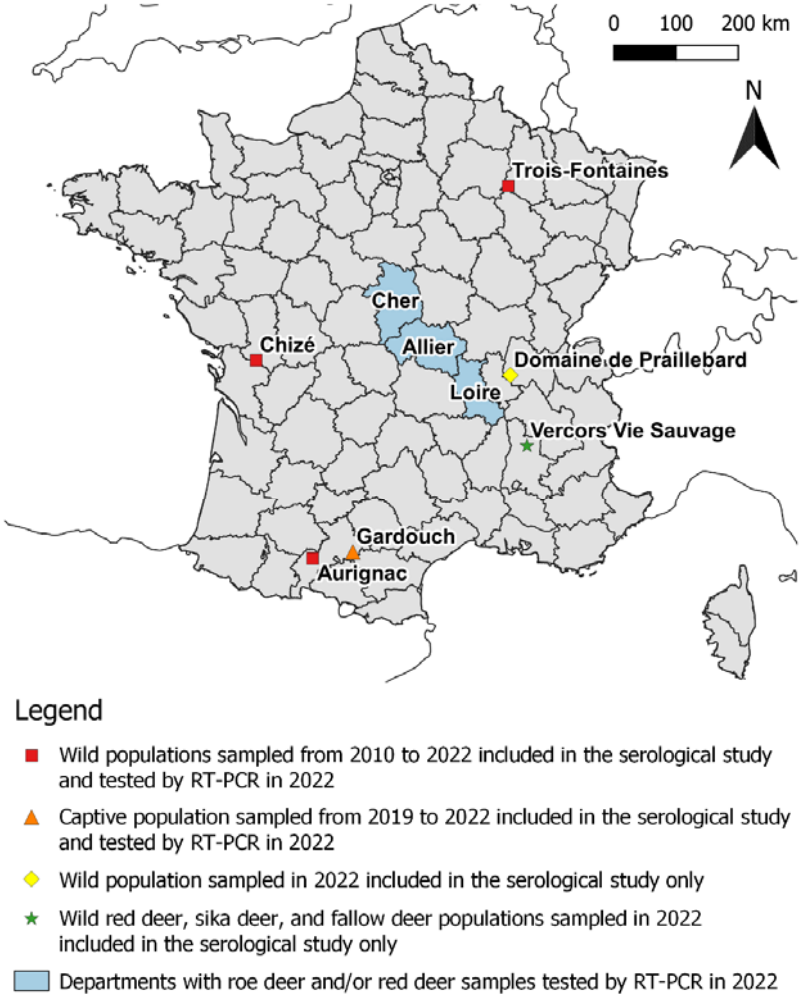

Figure 1. Locations of deer (Cervidae) populations sampled for the sera used in the SARS-CoV-2 serological study and the nasal swabs used in the SARS-CoV-2 RNA RT-PCR screening.

Table 1. Summary of the sampling scheme and the SARS-CoV-2 serological results for roe deer (*Capreolus capreolus*) and other cervids for pre- (*i.e.* 2010-2020) and post- (*i.e.* 2021-2022) COVID-19 emergence, set in March 2020.

Location (sampling period)	Species	Number of distinct individuals	Number of samples (pre-covid/2021/2022)	Number positive results (pre-covid/2021/2022)	Seroprevalence % (pre-covid/2021/2022)
Trois-Fontaines (2010-2022)	Roe deer	539	942 (832/29/81)	17 (16/1/0)	1.80 (1.92/3.45/0.00)
Chizé (2010-2022)	Roe deer	557	965 (817/29/119)	23 (15/0/8)	2.38 (1.84/0/6.72)
Aurignac (2019-2022)	Roe deer	180	205 (97/49/59)	7 (4/2/1)	3.41 (4.12/2.04/1.69)
Gardouch (2019-2022)	Roe deer	9	18 (6/4/8)	0 (0/0/0)	0.00 (0.00/0.00/0.00)
Domaine of Praillebard (2022)	Roe deer	4	4 (0/0/4)	0 (-/-/0)	0.00 (-/-/0.00)
Vercors Vie Sauvage (2022)	Fallow deer	16	16 (0/0/16)	1 (-/-/1)	6.25 (-/-/6.25)
	Red deer	9	9 (0/0/9)	0 (-/-/0)	0.00 (-/-/0.00)
	Sika deer	5	5 (0/0/5)	1 (-/-/1)	20.00 (-/-/20.00)
Total	**Roe deer**	**1,289**	**2,134 (1,752/111/271)**	**47 (35/3/9)**	**2.20 (2.00/2.70/3.32)**
	All species	**1,319**	**2,164 (1,752/111/301)**	**49 (35/3/11)**	**2.26 (2.00/2.70/3.65)**

2.2. Initial ELISA serological screening

Serum samples were first screened using the ID Screen® SARS-CoV-2 Double Antigen Multi-species ELISA kit (ID Vet, France) and the optical density (OD) was measured spectrophotometrically at 450 nm (Safas MP96, Monaco) following the manufacturer's instructions. The seropositivity threshold was S/P% ≥ 60, with $S/P = \frac{OD_{sample} - OD_{negative\ control}}{OD_{positive\ control} - OD_{negative\ control}} \times 100$.

2.3. Confirmatory seroneutralization assay

To confirm or refute positive ELISA results, the corresponding samples were further analysed using seroneutralization as described in Pozzetto et al. (2021). Briefly, Murine leukemia virus-based pseudoparticles carrying a green-fluorescent protein (GFP) reporter pseudotyped with SARS-CoV-2 spike protein (SARS-CoV-2pp) were used to measure the neutralizing antibody activity in sera. A sample of *ca.* 10^3 pseudoparticles was incubated with a 1/100 dilution of serum for 1 h at 37 °C before infection of the Vero-E6R cells (ATCC CRL-1586). The percentage of GFP-positive cells was compared to cells infected with SARS-CoV-2pp incubated without serum to set the percentage of neutralization. Anti-SARS-CoV-2 receptor-binding domain antibody was used as a positive control and a subset of 30 samples that were negative to ELISA tests from 2019 to 2022 were used as negative controls.

2.4. Direct detection

Nasal swabs (n = 244) were analysed using reverse transcriptase polymerase chain reaction (RT-PCR) technique. In an experimental study on white-tailed deer, RT-PCR on nasal swabs allowed to detect SARS-CoV-2 viral RNA at least 22 days post-infection, while infectious virions could only be detected for up to six days post-infection (Martins et al. 2022). Briefly, automated extraction instruments (KingFisher Flex 96 instrument by Thermo Fisher Scientific, Waltham, MA, USA, or the Auto Pure 96 instrument by Allsheng Instruments CO, Hangzhou, China) were used to extract viral RNA from swabs using the

BioExtract Premium Mag (Biosellal, Dardilly, France). Subsequently, SARS-CoV-2 RNA detection was performed by qRT-PCR with the Bio-T kit TriStar COVID-19 (Biosellal, France), which targets the envelope gene (E) of Sarbecoviruses (among which SARS-CoV-1, SARS-CoV-2), and RNA-dependent RNA polymerase (RdRp) in the Orflab gene, according to the manufacturer's instructions. The AriaMx Real-Time PCR Thermal Cycler System (Agilent Technologies, Santa Clara, CA, USA) was used for amplification. The condition consisted of 1 cycle of 20 min at 50 °C, 1 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, and 45 s at 60 °C. The results were analysed using the Agilent Aria 1.6 software, in which a cycle threshold value (Ct-value) < 40 for all target genes was defined as a positive result.

2.5. Analysis of seropositivity in roe deer

We analysed the distribution of ELISA results to better understand the origin of seropositive signals. First, under the hypothesis of SARS-CoV-2 emergence in roe deer populations following introduction from humans, a higher seroprevalence was expected in the post-COVID-19 human emergence period compared to the pre-COVID human emergence period. To test for this hypothesis, we compared the seroprevalences observed during two periods: either before COVID-19 emergence in France (pre-COVID-19 emergence: 2010-2020, n = 1,752, 'pre-Cov' hereafter) or after COVID-19 emergence (post-COVID-19 emergence: 2021-2022, n = 382, 'post-Cov' hereafter), for all populations together, with Chi-square tests (alpha = 0.05). We used samples of the first period as controls to test for potential cross-reaction with other coronaviruses circulating in these populations. We included the year 2020 in the pre-emergence period because sampling occurred between December 10th 2019 and March 12th 2020, before the virus spread across France and the lockdown was enforced on March 17th (Bernard-Stoecklin et al. 2020).

However, because animal manipulation was reduced in 2021 as a consequence of the implementation of social restrictions during the pandemic, the virus could have emerged in

roe deer populations later than 2021 (*i.e.* in 2022). Thus, considering a possible delayed exposition of the roe deer population, we ran a second test comparing the 2010-2021 *v.* 2022 periods. We thus defined two different periods: either before a delayed COVID-19 emergence in France (pre-delayed COVID-19 emergence: 2010-2021, n = 1,863, 'pre-dCov' hereafter) or after a delayed COVID-19 emergence with relaxed social restrictions (post-delayed COVID-19 emergence: 2022, n = 271, 'post-dCov' hereafter).

Serological results were further analysed to identify individual and population factors related to ELISA seropositivity. In this aim, we used 902 samples from TF, 866 from CH, and 202 from AU, corresponding to individuals of known age and sex. A binomial Generalised Linear Model (GLM) was used to model individual serological status. First, seropositivity could be cumulative with age, leading to autocorrelation with time in seropositive status. Thus, the probability that an individual with a positive result is also tested positive a following year was compared to the probability of an individual being positive a single year using a binomial test ($P(positivetwice|positive)$ *v.* $P(positive|tested)$, alpha = 0.05). Then, several potential explanatory variables were included in the GLM to explain individual serological result. To test for a contrast in seroprevalence after the COVID-19 emergence, pre-Cov and post-Cov periods as well as pre-dCov and post-dCov periods were included as distinct explanatory factors in the GLM but we excluded the possibility to retain both variables simultaneously. Seroprevalence may also have changed linearly over time for other reasons (*e.g.* environmental condition variation, forest management), so the temporal trend was also tested by using the sampling year as a linear integer (2010-2022). Seroprevalence was also expected to increase with age in the case of a cumulative effect due to antibodies persistence. This effect was tested adding age as a continuous variable (linear integer, 1-16 years of age). In TF and CH, age is precisely determined by observation of the dentition eruption of yearlings (Hewison et al. 1999) so that only individuals caught during their first

winter are known-aged individuals. For AU, the exact age was not known for all individuals, instead, age was estimated using five age classes through animal dentition observations (< 1, 1, 2-3, 4-5 and ≥ 6 years of age). In this case, the youngest possible age was considered (*e.g.* for an individual of the 4-5 years age class, it was considered as a 4-year-old individual) so that age was treated as a continuous variable as in TF and CH. As sex is also known to potentially influence susceptibility to infection (*e.g.* Channappanavar et al. 2017, Hewitt et al. 2024) this factor was also included in the models. One can also expect a higher increase in seroprevalence in enclosed populations with tight contacts with humans because of the follow-up research program. Population was thus included in the models in interaction with the COVID-19 emergence periods. As the effect of age can differ between sexes as observed in white-tailed deer (Caserta et al. 2023), models included the age:sex interaction. Because population-specific characteristics can act differently on individual condition with advancing age, as it is for example the case for immunosenescence patterns that differ between TF and CH (Cheynel et al. 2017, Lalande et al. 2024), models included an age:population interaction. Finally, because temporal trends can differ among populations, the population:year interaction were also included in the models.

Model selection was then performed to select the best model based on the Akaike Information Criteria corrected for small sample sizes (AICc): models with ΔAICc < 2 were kept and the significance of the variables included in those retained models was evaluated using type III ANOVAs. To evaluate the significance of the difference between groups for significant categorical variables, *post hoc* between groups paired comparisons were conducted using binomial tests. All statistics were done with R version 4.2.2 (R Core Team 2023) in Rstudio 2023.03.0 (Posit Team 2023), and with the *car* 3.1-2 (Fox and Weisberg 2019), *MuMIn* 1.47.5 (Bartoń 2023), *readr* 2.1.4 (Wickham et al. 2023) and *visreg* 2.7.0 (Breheny and Burchett 2020) packages.

3. Results

3.1. Serological results

A total of 47 out of 2,134 ELISA tests (2.20% [95%CI: 1.62-2.92%]) performed on roe deer sera had positive results for anti-SARS-CoV-2 antibodies against the N-protein, including samples collected during both the pre-Cov and post-Cov periods (Table 1). The S/P% values of positive results ranged from 60.7 to 824.4% and no evident bimodal distribution was observed, as expected when a proportion of the population have been exposed to a pathogen and displays a high antibody concentration. Although seroprevalence was higher in the post-COVID-19 emergence period (delayed or not) than before SARS-CoV-2 emergence, this difference, when considering all roe deer populations together, was not statistically significant (pre-Cov *v.* post-Cov Chi² test: $\chi^2 = 1.41$, df = 1, $p = 0.24$; pre-dCov *v.* post-dCov Chi² test: $\chi^2 = 1.26$, df = 1, $p = 0.26$). Additionally, one roe deer tested twice three weeks apart during the same year was positive for each sample with a decline from 328% to 120% in observed S/P%. Only the first observation was kept for the subsequent analyses. Among the sera from other cervids inhabiting the VVS reserve, ELISA tests performed on one fallow deer out of 16 and one sika deer out of five yielded seropositive results (Table 1). SARS-CoV-2 seroneutralization assays were performed on nine positive and 15 negative roe deer samples of the post-COVID-19 emergence period, and on four positive and 15 negative roe deer samples of the pre-COVID-19 emergence period. All seroneutralization assays, including the 13 carried out on ELISA-positive samples, yielded negative results.

3.2. Direct virus detection

RT-PCR carried out on nasal swabs collected during winter 2022 on 205 captured roe deer (of which 10 were positive with the ELISA assay) and 36 hunted roe deer and three hunted red deer, did not reveal any positive result.

3.3. Analysis of seropositivity in roe deer

The 47 positive sera originated from 46 individuals from CH, TF, and AU (see Table 1), including 28 individuals sampled and tested only once, seven tested twice, six tested thrice, four tested four times and one tested five times. During the follow up of the 19 positive individuals tested multiple times, both appearance and disappearance of antibodies were evidenced, as 13 seronegative individuals were found seropositive in the following years (six on the next year, seven two years or more later), while 11 negative results were observed on samples that were previously positive (four on the next year, seven two years or more later). Only one individual was tested positive twice during two different years (2016 and 2018) but no sample was available in-between (*i.e.* 2017). 20 positive results were from individuals in their first year. The probability that an individual was positive two different years was not different from expected by chance based on the observed prevalence (binomial test: $p = 1$). So, seropositivity events (47 sera in 46 individuals) were considered as independent in the following analyses.

Individual seropositivity was analysed in 1,970 samples obtained on 1,141 individuals for which population, sampling year, age and sex were known in AU, CH and TF populations. The models retained according to the Akaike Information criterion (AICc)-based selection procedure (ΔAICc $\leq$ 2) are presented in Table 2. The most parsimonious model of seroprevalence included the positive linear effect of age ($\beta \pm SE = 0.31 \pm 0.24$), the delayed COVID-19 emergence variable (dCov: 0.80 ± 1.11) and the effect of population (AU [intercept]: -5.11 ± 1.39, CH: 3.33 ± 1.46, TF: -13.48 ± 748.18). We also found a significant interaction between the delayed emergence variable and population (AU [intercept]: -5.11 ± 1.39, CH: -2.33 ± 1.21, TF: 13.84 ± 748.18), showing a higher seropositivity in CH in 2022 compared to the 2010-2021 period (Figure 2). We evidenced a significant interaction between age and population (AU [intercept]: -5.11 ± 1.39, CH: -0.60 ± 0.27, TF: -0.30 ± 0.26), showing a decrease in seropositivity with age in CH only (Figure 3). Note that the variable

comparing pre-Cov and post-Cov periods was never retained in the higher-ranked models (Table 2). In contrast, those models all included the pre-dCov *v.* post-dCov periods variable. In these models, the effect of the population and its interaction with the delayed emergence variable and with age were also always retained.

Finally, we conducted *post hoc* between groups paired comparisons on the retained model interactions. We found that the seroprevalence was higher during the post-dCov (*i.e.* 2022) in CH (pre-dCov: 1.60% [95%CI: 0.83-2.79%], post-dCov: 6.78%, [2.97-12.92%], $p < 0.01$; Fig. 2), and decreased with age at CH, but not in TF and AU. Seroprevalence decreased from 4.50% [95%CI: 2.48-7.44%] during the first year of life to 0.68% [95%CI: 0.08-2.43%] in individuals aged 5 years old and more in average for both periods ($p < 0.01$; Fig. 3) but was more marked in post-dCov.

Table 2. Summary of seroprevalence models retained by the AICc-based selection procedure. Only models within 2 ΔAICc are presented.

Int	Age	Cov	dCov	Pop	Sex	Year	Age:Pop	Age:Sex	Cov:Pop	dCov:Pop	R^2	df	logLik	AICc	ΔAICc	weight
-5.11	**0.31**		**+**	**+**			**+**			**+**	**0.01**	**9**	**-191.62**	**401.33**	**0.00**	**0.47**
-132.89	0.31		+	+		0.06	+			+	0.01	10	-191.11	402.32	0.99	0.29
-5.28	0.32		+	+	+		+			+	0.01	10	-191.29	402.69	1.36	0.24

Int: intercept; Cov: COVID-19 emergence period (2010-2020 *v.* 2021-2022); dCov: delayed COVID-19 emergence period (2010-2021 *v.* 2022); Pop: population (Aurignac, Chizé, Trois-Fontaines); df: degrees of freedom; logLik: log-likelihood; AICc: Akaike Information Criterion corrected; ΔAICc: difference of AICc from the best model; weight: relative model weight. See text for further details.

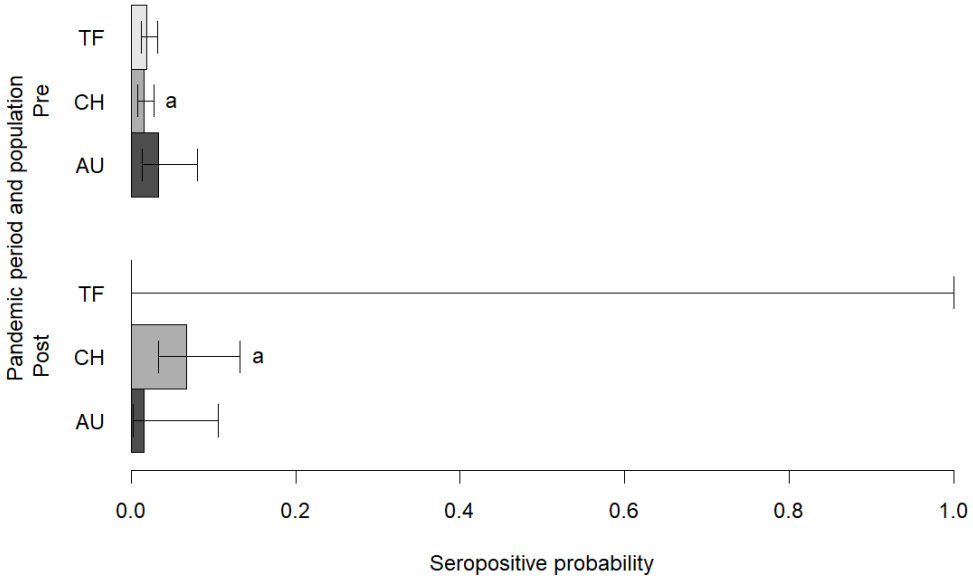

Figure 2. SARS-CoV-2 seroprevalence of roe deer (*Capreolus capreolus*) population and the delayed COVID-19 emergence periods. The plot displays fitted seroprevalence with 95% confidence interval for Aurignac (AU), Chizé (CH) and Trois-Fontaines (TF) populations for the pre- (2010-2021; "Pre"; upper panel) and post-delayed COVID-19 (2022; "Post"; lower panel) emergence periods; "a" indicates a significant difference ($p < 0.05$) in *post hoc* between groups paired comparisons.

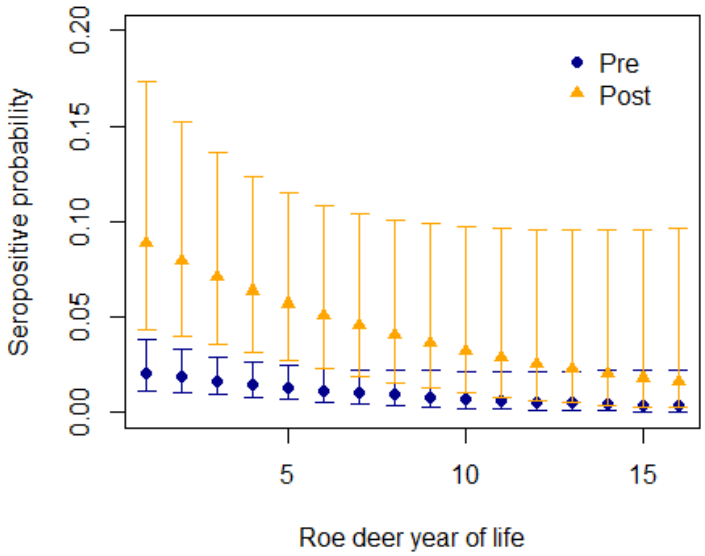

Figure 3. SARS-CoV-2 seroprevalence of roe deer (*Capreolus capreolus*) in the population of Chizé (CH) as a function of age and delayed COVID-19 emergence periods. The plot displays fitted seroprevalence in CH during the pre- (2010-2021, 'pre', blue dots) and post-delayed COVID-19 (2022, 'post', orange triangles) emergence periods (see text for more details) with 95% confidence intervals.

4. Discussion

This study is the first long-term individual monitoring of serological status of wild-living cervids for SARS-CoV-2. Our survey intended to detect, at the individual or population levels, an increase in seropositivity or seroprevalence following the emergence of SARS-CoV-2 in France.

Interpreting serological results in terms of viral infection is not straightforward. SARS-CoV-2 ELISA tests indicated an overall seroprevalence of 2.20% in roe deer. We also observed positive results in other cervids from VVS despite a low sample size (one out of 16 fallow deer and one out of five sika deer). However, all sera submitted to the seroneutralization assay, and all swabs tested for direct detection of the virus by RT-PCR in 2022 gave negative results. This indicates that sarbecoviruses, among which SARS-CoV-2, are unlikely to circulate in the studied populations of roe deer.

The absence of confirmation of SARS-CoV-2 antibodies by seroneutralization assay is consistent with other studies conducted in Germany, Austria and the UK on roe deer, red deer, fallow deer, sika deer, Reeves's muntjac (*Muntiacus reevesi*), and Chinese water deer (*Hydropotes inermis*) (Holding et al. 2022, Wernike et al. 2022a, Moreira-Soto et al. 2022, Encinas et al. 2023). Negative seroneutralization assays despite positive ELISA tests have also been reported in wild boars (*Sus scrofa*), red foxes (*Vulpes vulpes*) and jackals (*Canis aureus moreoticus*) (Jemeršić et al. 2021, Wernike et al. 2022a). It can be explained by an inadequate specificity of the ELISA test allowing cross-reaction with antibodies directed to other closely-related viruses (Krüttgen et al. 2021). Also, while ELISA used in this study detect antibodies targeting SARS-CoV-2 nucleoprotein, the seroneutralization assay detects neutralizing antibodies targeting spike glycoprotein. Previous studies showed a poor correlation between antibodies targeting nucleoproteins and neutralizing antibodies in SARS-CoV-2-infected patients (Legros et al. 2021). This could also explain why a serum can score

positive in a nucleoprotein-based ELISA assay, but negative in a seroneutralization assay. Such results underline the importance of not relying on ELISA tests alone before drawing any conclusions.

Nonetheless, these positive results suggest that the ELISA test used likely cross-reacts with one or several other coronaviruses circulating in the studied roe deer populations. Viruses close to bovine coronaviruses (BCoVs) have already been found in red deer and sika deer (Amer 2018), and are potential candidates that could have been detected by our ELISA analyses. Also, the circulation of phylogenetically close coronaviruses transmitted by humans to cervid populations, such as HCoV-OC43 or another still unknown coronavirus in cervid populations is also possible, as suggested elsewhere (Vijgen et al. 2006, Wernike et al. 2022a). More investigations are required before drawing any firm conclusion, for instance by using more specific tests to confirm or infirm the presence of BCoVs antibodies in ELISA positive roe deer sera (Ulrich et al. 2020).

Positive serological results were observed in all populations of roe deer, except DP and GA for which only four and 18 sera were tested, respectively. If a virus of bovine origin is involved, one would expect a lower seroprevalence in the two isolated roe deer populations of CH and TF compared to the free-roaming roe deer population of AU exposed to cattle, and higher seroprevalence in captive roe deer in GA. Our results do not show such pattern and in principle would not support the BCoVs hypothesis. However, other possibilities would include (*i*) long-lasting BCoVs infections maintenance in the isolated populations since the enclosure of the areas, (*ii*) the introduction of BCoVs by infected animals entering the enclosure by some gaps in the fences, which is possible for roe deer or another susceptible species like wild boar, or (*iii*) introduction of a human coronavirus in cervid populations. Note also the seropositivity of a fallow deer and a sika deer, which were not in such close contact with human than studied roe deer, supports the idea of at least one cross-reacting coronavirus

in wild cervid populations. Finally, as the virus(es) responsible of the observed seroprevalence is unknown, one cannot exclude the possibility that distinct viruses circulate in the different populations.

When studying more closely the spatio-temporal and individual factors of seropositivity in the three roe deer populations with a sufficient sample size (AU, CH and TF), we detected a significant statistical interaction between the pre- and post-COVID-19 delayed emergence periods and populations. Although these results must be interpreted cautiously because of the low explained variance by the models, seroprevalence increased in CH from about 1.60% to 6.78% in 2022 while it fell to 0.00% in TF and did not change substantially in AU. We found no evidence that seroprevalence increased with age, and even found that seroprevalence decreased with age in CH. Furthermore, among 11 positive individuals that were tested the following years, only one was found seropositive twice. Other individuals showing seropositive status were tested negative during the following years, including four individuals tested only one year later. Together, these results are consistent with a short persistence of the antibodies (less than one year), but for at least several weeks (>3). Although a rapid decline of humoral response has been described, antibodies after natural SARS-CoV-2 infection in humans can persist for more than a year (Ghasemi et al. 2022, Sarjomaa et al. 2022). In white-tailed deer, it should last at least 13 months (Hamer et al. 2022), but persistence in other species is not well known. Thus, according to available data in white-tailed deer, as persistence time is expected to be longer and juveniles are not expected to have a higher seroprevalence, our observations are not consistent with a SARS-CoV-2 emergence in the studied roe deer populations (Hamer et al. 2022, Hewitt et al. 2024). Data from coming years may clarify these temporal trends in the different populations. Nonetheless, without formally identifying the responsible virus(es), observed patterns are difficult to explain.

Transmission of the SARS-CoV-2 virus from human to roe deer is thus unlikely to have happened to date in the studied populations, contrary to what has been observed among white-tailed deer populations in North America (Chandler et al. 2021, Hale et al. 2022, Kuchipudi et al. 2022). Our results confirm the results of previous studies evidencing an absence of contact between the most common European cervids and the SARS-CoV-2 virus (Holding et al. 2022, Wernike et al. 2022a, Moreira-Soto et al. 2022), except in densely populated areas (Encinas et al. 2023). Indeed, roe deer is not living in peri-urban areas as frequently as white-tailed deer, and thus contact rate between roe deer and human is expected to be lower than for white-tailed deer. Roe deer is also not as close to white-tailed deer relative to their ACE2 receptor (which interacts with the SARS-CoV-2 spike protein) and likely less susceptible to SARS-CoV-2 infection (Damas et al. 2020). Despite the low probability of roe deer, or other European cervids, to serve as a reservoir host for SARS-CoV-2, the circulation of one or several other coronaviruses, maybe BCoVs, in roe deer populations might raise the question of a possible virus cross-recombination leading to a new virus variant. Further studies should be carried out to identify coronaviruses circulating in wild cervids and domestic ruminants, and to continue testing roe deer populations to enable track temporal trends. Maintaining serological surveillance and implementing efficient virological surveillance to detect viruses in wildlife is necessary to evaluate the possibility of transmission between human and other domestic or wild animals (Wernike et al. 2022b).

Data and code availability

All data and codes used for the present analyses have been deposited on the CIRAD dataverse and are publicly available: https://doi.org/10.18167/DVN1/AR6UDN

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Ethics and authorisations

The research presented in this article was done according to all institutional and/or national guidelines. The protocol of capture and blood sampling of roe deer is under the authority of the *Office Français de la Biodiversité* (OFB) and was approved by the Director of Food, Agriculture and Forest (Prefectoral order 2009-14 from Paris). All procedures were approved by the Ethical Committee of Lyon 1 University (project DR2014-09, June 5th, 2014). The Trois-Fontaines population is part of the long-term Studies in Ecology and Evolution (SEE-Life) program of the CNRS. The data from Aurignac population came from a long-term study approved by ethical committee n°115 and by French Ministry of Higher Education and Research (Apafis#39320)

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