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1 **Wild ungulates as sentinel of BTV-8 infection in piedmont areas**

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14

15 **Abstract:**

16 Bluetongue caused by the serotype 8 virus (BTV-8) appeared for the first time in BTV free areas in
17 northern Italy in 2008. The presence of domestic animals outbreaks, abundant wild ungulates
18 populations, and ongoing regional BTV control plans, made this area interesting to evaluate the
19 role of wild ruminants in BTV-8 epidemiology. We analyzed spleen samples from hunted red deer
20 (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and Alpine chamois (*Rupicapra rupicapra*) by
21 quantitative RT-PCR. Samples were collected from 2008 to 2011 in two provinces of Piedmont
22 region. BTV-8 was detected in all ungulate species, confirming their receptivity to the infection.
23 However, the viral load in the positive specimens was low, and decreased from 2008 to 2011.
24 These results, together with the extinction of the epidemic following a regional livestock
25 vaccination campaign, lead to hypothesize that wild ungulates were an epiphenomenon and that
26 they had not an important role in the domestic transmission cycle of BTV-8 in the area. In spite of
27 this, wild ruminants appear to be good sentinels of BTV circulation and their monitoring could be
28 useful for surveillance in piedmont areas.

29

30

31 **Introduction**

32 Bluetongue (BT) is an infectious disease of ruminants caused by a RNA virus (BTV) belonging to the
33 *Reoviridae* family, genus *Orbivirus*. The virus may infect various ruminant species, although clinical
34 disease and mortality are generally associated with sheep (Erasmus, 1990). It is characterized by a
35 high genetic variability, and 26 serotypes (including the new Toggenburg and Kuwait serotypes;
36 Chaignat et al., 2009; Maan et al., 2011) have been recognized so far. Midges of the genus
37 *Culicoides* (Diptera: *Ceratopogonidae*) are the vectors of BTV, and their distribution determines
38 the geographic diffusion of the disease (Maclachlan et al., 2011).

39 BT is a reportable disease with high socioeconomic impact, affecting the international trade of
40 animals and animal products (Saegerman et al., 2008). It was considered an exotic disease with
41 probable African origin until 1998, when the virus began its spread in Europe (Eschbaumer et al.,
42 2009). After 2006, some BTV serotypes expanded across the 45° parallel (Saegerman et al., 2008).
43 The most important outbreaks in central and northern Europe were caused by BTV-8. This
44 serotype showed peculiar characteristics, including an unexpected virulence in cattle (Thiry et al.,
45 2006).

46 In Italy serotypes 2, 4, 9, and 16 have been reported since 2000 in central and southern regions. In
47 2008 BTV-8 appeared in the north, in Veneto and Piedmont regions, and in Sardinia, while BTV-25
48 was present in Lombardia region, close to the Swiss borders (Sozzi et al, 2009).

49 Piedmont region was a previously BTV free area in north-western Italy (Fig. 1). BTV-8 infection was
50 firstly reported in a cow during October 2008 in Cuneo province (Fig. 2A), from a sentinel farm
51 included in the Italian BTV control program (Caporale and Giovannini, 2010). The farm had a mixed
52 breeding of cows, sheep and goats, and two bulls had been introduced in 2004 and 2007 from
53 France. Seven days after the removal of the infected cow, a 6 months-calf born in the same farm
54 was found positive. In the following months of 2008, other infections caused by BTV-8 were

55 recorded in neighboring areas, for a total of 16 positive farms. In 2009 and at the beginning of
56 2010, ten new positive farms were discovered in Turin and Asti provinces[1] (Fig. 2A). Most BTV-8
57 outbreaks occurred at the foothills of the Alps, in areas characterized by woodland and low
58 zootechnical density, where domestic animals are sometimes free-ranging. In Piedmont, culicoides
59 of the *Obsoletus* and *Pulicaris* complexes are present (Goffredo et al., 2004).

60 Various measures to limit the spread of the disease were adopted by the Piedmont veterinary
61 services, in relation to a local risk analysis, including restrictions to the movement of susceptible
62 animals and the implementation of two vaccination campaigns in domestic ruminants. The
63 performed activities prevented the damages of the disease, maintained the trade flows and
64 reduced the spread of the infection, with the last goal of disease eradication[2].

65 Different hypothesis were proposed to explain the introduction of BTV in Piedmont. First, the
66 importation of infected animals from France; indeed, around 180000 heads of cattle are imported
67 every year in Piedmont, mainly to fattening farms[3] . However, also the free movements of infected
68 wild animals and vectors from neighboring countries (France and Switzerland) could be implicated.
69 BTV-8 was indeed widespread in France during the late summer of 2008, and cases had been
70 reported in areas close to the Italian borders. The existence of interconnected domestic and wild
71 cycles that could account for the maintenance of BTV has been hypothesized (Ruiz-Fons et al.,
72 2014).

73 In this study, we employed RT-qPCR to analyze tissue samples from red deer, roe deer and
74 chamois captured during the 2008-2011 hunting seasons in Piedmont, to evaluate the role of
75 wildlife during the BTV-8 epidemics in the region. Determining such role could be very important
76 for the implementation of surveillance plans and to face future BTV epidemics.

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78 [1] http://www.regione.piemonte.it/sanita/cms/documentazione/category/96-2011.html
79 [2] http://www.izsto.it/index.php/report-dati-sanitari/sanita-animale/bovini/blue-tongue
80 [3] National husbandry register. Statistics.
81 http://statistiche.izs.it/portal/page?_pageid=73,12918&_dad=portal&_schema=PORTAL

Materials and methods.

Study area.

Piedmont is located in north-western Italy (44°23’0’’ N, 7°33’0’’ E; Fig.1). It is an Alpine region characterized by an important livestock farming system. According to 2013 statistics, cattle heads are estimated at around 778000 (13% of Italy) and small ruminants at 188000 (2% of Italy[2]). Our study focused in Cuneo and Torino provinces (Fig. 1), in which the main domestic BTV-8 outbreaks occurred in 2008-2009 (Fig. 2a). Sampling was performed in hunting districts in the alpine valleys. In these areas, livestock is usually brought to pasture during summer months. Animals were captured at an altitudinal range comprised between 300 and 1600 m a.s.l. (above sea level). Moreover, hunted red deer from an enclosed regional natural park in the periurban area of Torino (La Mandria, 265 m above sea level; Fig.1) were sampled. Livestock breedings are located in close proximity of the park walls.

The regional authorities coordinate wildlife censuses and control hunting in the region. The hunting quotas, based on ungulates numbers which are compatible with the agro-sylvatic and pastoral activities, were set at 0.5-1 red deer, 5-10 roe deer, and 6 chamois per 100 ha[3]. A different management is adopted in 'La Mandria' park, in which the red deer density is at present estimated at 14 red deer per 100 ha.

Animal tissues collection.

Samples were collected during the hunting seasons in the period March 2008 - June 2011 from red deer (*Cervus elaphus*), Alpine chamois (*Rupicapra rupicapra*) and roe deer (*Capreolus capreolus*).

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[3]www.regionepiemonte.it

107 Ungulates were brought from hunters to hunting lodges and spleen were collected and stored at -
108 20C°until analysis. Spleen was the target organ of chooses for maximizing the likelihood of
109 detecting BTV RNA by molecular analysis (López-Olvera et al., 2010; Worna et al., 2010; Rossi et
110 al.,2014), since sample collection was performed in the field and it was occasionally carried out by
111 hunter. Species, sex, age, location and hunting date of animals were recorded. The animals were
112 classified as juveniles (less than 1 year old) or adults (equal or more than 1 year old). Aging was
113 based on tooth replacement and corn.
114
115 *Molecular analyses.*
116 *RNA extraction.* RNA was extracted by using Trizol reagent (Invitrogen) from 100 ug tissue, in
117 accordance with the manufacturer's protocol, and quantified with NanoDrop 2000 instrument
118 (Thermo Scientific).
119 *Real time RT-PCR* (RT-qPCR). The primers targeting the NS3 gene of BTV were designed based on
120 ClustalW multiple alignment of GeneBank reference sequences of BTV genotypes circulating in
121 Italy or at risk of introduction (1, 2, 4, 8, 9 and 16). The RT-qPCR assay was performed using 100ng
122 of RNA extracted from the samples. The 25µl reaction mix contained 5ul of RNA extract in RNase
123 and DNase-free water, 2XQuantiFast RT-MIX SYBR Green (Qiagen), 0.03 mM concentrations of
124 both primers (forward primer: 5'-TAT CCG GGC TGA TYC RAA GG -3'; and reverse primer: 5'-GCG
125 TAC GAT GCG AAT GCA GC-3'). The mix of primers and template was denatured at 95°C for 5min
126 and subsequently added at the reaction mix. Cycling condition included: retrotranscription at 50°C
127 for 30min, denaturation at 95°C for 5 min; 50 cycles of 95°C for 10s, 60°C for 30s. Post-
128 amplification melting temperature (Tm) analysis from 50 to 95°C at 0.5°C increments was
129 conducted to determine specific NS3 product (Tm ~ 83.6 °C) (Applied Biosystems 7300 Real Time
130 PCR System). The Applied Biosystems detection software was used to determine threshold cycle

131 (Ct) values, Tm, and the standard curve. Negative controls, including RNase and DNase-free water
132 and nucleic acid extracts from sure negative samples, were used to determine any possible cross-
133 reactivity or contamination (false-positive results). RNA from a BTV-2 positive sample was used as
134 positive control. All experiments were run in duplicate and were replicated twice. ATPase gene
135 amplification was included as a control, in order to detect any inhibition in the BTV detection.
136 Some positive samples were sequenced to confirm the results (BMR genomics, Padova, Italy). The
137 chromatograms of the nucleotide sequences were submitted to BLAST analysis.
138 *Standard curve.* For calibration of the RT-qPCR assay, we cloned the BTV-8 amplicons into pCR-XL-
139 TOPO cloning vector (Life Technologies). The identity of the insert was confirmed by sequencing.
140 After plasmid preparation, we generated a standard curve with known copy numbers of NS3-RNA
141 transcripts from the plasmid, containing the NS3 fragment obtained by PCR. The Riboprobe kit
142 (Promega) was used to produce the standard transcripts, following the 'Large Amount RNA
143 Synthesis protocol' provided by the manufacturer. Transcribed RNA was DNase treated, purified
144 using an RNA clean-up protocol (RNeasy MinElute Cleanup Kit, Qiagen) and extracted using Trizol
145 (Life Technologies). The purified RNA was quantified by use of Nanodrop and the corresponding
146 copy number was calculated. Calibration curves were generated by amplification of 100 fold serial
147 dilutions of 10^7 to 10^1 RNA copies per reaction. Dilutions were also used to test the sensitivity
148 limits of the assay: a log-10 titration series of in vitro transcribed RNA indicated that less than 10
149 copies of BTV NS3 gene segment ssRNA could be detected.
150
151 *Statistical analysis:*
152 Prevalence of PCR-positive results were calculated, with 95% exact binomial confidence intervals
153 (95%CI), per hunting season, animal species, sex and study area. The hunting season was
154 considered as starting from September each year. The Fisher's Exact test was used to study the

155 association among categorical variables. Mean and standard deviation of Ct levels of positive
156 samples were calculated by hunting season year. Analyses were performed by R software (R
157 Development Core Team 2008).
158
159 **Results:**
160 A total of 235 animals were sampled: 102 red deer, 78 roe deer, and 55 chamois. Most of the
161 samples were collected during the hunting seasons 2009-2010 (n=135; from 48 red deer, 43 roe
162 deer, 44 chamois) and 2010-2011 (n=84; from 51 red deer, 24 roe deer, 9 chamois). Other 16
163 samples were collected during the 2008-2009 hunting season (from 3 red deer, 11 roe deer, 2
164 chamois).
165 Due to the convenience sampling, samples distribution by species was not homogeneous in the
166 study areas: most of red deer samples (92.2%) were collected in Torino province, while the
167 majority of roe deer (91.0%) and chamois (89.1%) were sampled in Cuneo.
168 Spleen samples were submitted to RT-qPCR analysis. BTV NS3 gene was detected in 15.7% of the
169 animals (n=37, 95% CI 11.3-21.0): 21 red deer, 12 roe deer and 4 chamois. The threshold cycle (Ct)
170 RT-PCR values ranged between 23.15 and 35.2, with a mean Ct value of 30.4±2.6. The melting
171 curve analysis revealed that positive samples belonged to BTV serotype 8. To confirm the results,
172 three samples from red deer were sequenced: one from Torino valley (hunting season 2008-2009),
173 one from Cuneo valley and one from 'La Mandria' park (hunting season 2009-2010). The
174 sequences showed a similarity of 99.6%, 98.9% and 99.5% to BTV-8 respectively.
175 Infection prevalence by animal species, sex, age, hunting season, and province of collection, is
176 shown in Table 1. No difference in the BTV prevalence was detected among ungulate species
177 (Fisher Exact test; p=0.09). However, red deer were the most infected species in all sampled areas.
178 Indeed, in Cuneo province, three out of eight examined red deer were infected (37.5%; 95%CI: 8.5-

179 75.5), whereas only 8.2% (95%CI: 2.3-19.6) of chamois and 16.9% (95%CI: 9.0-27.7) of roe deer
180 resulted positive. In Torino province, only red deer were found infected; however, only a small
181 number of chamois (n=7) and roe deer (n=6) was examined.
182 The age class and sex of the animals were not significantly associated with PCR positivity (p=0.15
183 and p=0.8 respectively).The infection prevalence was significantly different among hunting years
184 (p=0.034), with BTV prevalence decreasing from 2008 to 2011 (Table 1). Moreover, positive
185 samples from 2008-2009 showed lower mean Ct values (28.5±2.7) compared to the other hunting
186 seasons (30.6±2.7 in 2009-2010, and 30.6±1.8 in 2010-2011). Most of the infected animals (n=26)
187 were hunted during late summer-autumn, but some positivities were also registered in wintertime
188 and early spring (December to June).
189 BTV prevalence was similar in the two Piedmont provinces (Table 1). When considering red deer
190 only, no difference in the infection prevalence between La Mandria (25%; 95%CI: 14.3-38.4) and
191 the Alpine valleys (15.2%; 95%CI: 6.3-28.9) was recorded (p=0.3), while a significant difference
192 (p=0.036) was found when considering all animal species (prevalence in the Alpine valleys: 12.8%;
193 95%CI: 8.3-18.6). Positive animals were recorded in Cuneo from April 2008 to October 2010, and
194 were captured in municipalities located from 480 m to 1650 m a.s.l. In Torino province, the
195 positive animals were captured from October 2009 to October 2010 in municipalities located
196 between 260 and 1600 m a.s.l. Fig. 2 shows BTV-8 domestic outbreaks (A), and municipalities in
197 which wild ungulates were tested (B) in 2008-2010 in Piedmont. The geographic pattern suggests
198 a temporal association in the same areas between domestic and wildlife positivities, with positivity
199 in wildlife following with one-year delay the domestic outbreaks. No animals captured between
200 October 2010 and July 2011 (n=26) resulted infected.
201
202 **Discussion**:

203 Our study confirms the wild ungulates receptivity to BTV-8 infection (García-Bocanegra et al.,
204 2011; Linden et al., 2010; Rossi et al., 2010, 2013).
205 As reported in other studies, we detected a low viral load in the positive spleens, which could
206 indicate a very low or absent viral load in blood and skin (Worna et al., 2010). Our positive
207 ungulates were probably not able to infect biting midges, at least in the moment they were
208 hunted. Indeed, experimental infections in red deer indicated that, although the BTV RNA is
209 detectable up to 112 days post infection (dpi), the isolation from skin samples was only successful
210 during the viraemic peak at 14 dpi, which corresponds to the peak of BTV RNA detection by RT-
211 PCR in blood. This demonstrated that the infectious agents can be transmitted to the midges only
212 during the viraemic peak of infection (López-Olvera et al., 2010). BTV-8 experimental infection in
213 calves, where the virus could be detected up to 157 days, showed that real time RT-PCR remains
214 positive although no circulating virus is detectable in the peripheral circulation (Di Gialleonardo et
215 al., 2011). RT-PCR is thus a valuable method to estimate recent BTV infections in wildlife (Rossi et
216 al., 2013), but it is not suitable to assess the time at risk in which culicoides can be infected, due to
217 the discrepancy between the time in which virus isolation is possible and RNA is amplified by RT-
218 qPCR (Worwa et al., 2010). Moreover, the minimal level of viraemia in a host, necessary to infect
219 the *culicoides*, is not known. Although virus titers in blood lower than 10^3 $TCID_{50}$ (median tissue
220 culture infective dose)/ml have traditionally been considered a safe threshold, insects acquiring
221 BTV from animals with lower viraemic titers have been reported (Savini et al., 2008). However, a
222 high BTV-8 viral concentration in the bloodmeal of artificially fed midges resulted in absence of
223 infection after the insect extrinsic incubation period (Del Rio López et al., 2012).
224 We observed a slightly higher BTV prevalence in red deer compared to the other species, although
225 this difference was not significant. In France, high RT-PCR prevalence for BTV1 and BTV-8 were
226 reported in red deer (77.8% and 37.8% in 2008 and 2009, respectively), while BTV-8 was not

227 detected in three roe deer and one Alpine chamois (Rossi et al., 2013). Also serological studies
228 report the highest seroprevalences in red deer (up to 66.3%) and fallow deer (*Dama dama*; up to
229 50%), both belonging to the *Cervinae* subfamily (Corbière et al., 2012; García-Bocanegra et al.,
230 2011; Linden et al., 2008; Linden et al., 2010; Rossi et al., 2010 and 2013; Ruiz-Fons et al., 2008).
231 Roe deer were generally found sero-negative (Boadella et al., 2010; Corbière et al., 2012) or with
232 sero-prevalences up to 5.1% (Linden et al., 2010; Rossi et al., 2010; Ruiz-Fons et al., 2008). Scarce
233 information can be found for the Alpine chamois, which were found sero-negative by Corbière et
234 al. (2012) and Rossi et al. (2010). Antibodies to BTV were also detected in Europe in mouflons
235 (*Ovis aries musimon*), Spanish ibex (*Capra pyrenaica*) and ibex (*Capra ibex*) (Falconi et al., 2011).
236 In Europe, red deer is considered the most relevant wild ungulate host for BTV (Ruiz-Fons et al.,
237 2014). Indeed, red deer can maintain the viral RNA for long periods, remaining essentially
238 asymptomatic (Falconi et al., 2011), and populations are abundant. Areas with higher red deer
239 densities in Spain have been shown to display higher BTV prevalence (Garcia et al. 2009); high
240 animal densities can indeed favor the effective contact rate for the infected culicoides. In our
241 study areas, red deer populations have increased their abundance in the past decades. Although a
242 higher infection prevalence was detected in red deer living in a high density area (La Mandria)
243 compared to the Alpine valley populations, the difference in infection prevalence between the
244 different areas was not significant. The high infection prevalence in La Mandria could be due to
245 the close proximity to the park walls of some livestock breedings which were found positive to
246 BTV-8 in 2009, since infected insects can easily fly between the external area and the park. A
247 significant difference in infection prevalence was instead recorded when considering all animal
248 species, probably because of the lower prevalence in mountain ungulates such as the chamois, as
249 observed in France (Rossi et al., 2013).

250 Sex and age did not appear to be risk factors for BTV infection in our study. BTV association with
251 these factors are not consistent throughout literature (Ruiz-Fons et al., 2014).
252 We observed a progressive viral load reduction in the positive samples collected from 2008 to
253 2011. This could be associated to a lower viral circulation in Piedmont region, which occurred after
254 the 2009 and 2010 vaccination campaigns: the reduced infection burden in domestic animals
255 could have determined a consequent lower prevalence in biting midges and wildlife. Indeed, we
256 did not detect infection in ungulates after October 2010. The finding of positivities in early winter
257 and spring could be a result of persistent infections.
258 The temporal association between the positivity in domestic and wild animals is noteworthy.
259 Indeed, the first domestic outbreaks occurred in Cuneo in 2008 and in Torino in 2009, and we
260 found positive wild ungulates in Cuneo from 2008 onwards, and in Torino from 2009 onwards (Fig.
261 2). Wildlife infection thus followed the domestic animal infection trend. As observed in France
262 (Rossi et al., 2013), BTV was maintained in wild ungulates in periods characterized by negligible
263 livestock infections.
264 All these data may suggest that wild ungulates represented an epiphenomenon, and did not have
265 an important role in the domestic transmission cyle of BTV-8 in Piedmont. This thesis is confirmed
266 by the 'natural experiment' which occurred in the region one year after the end of the compulsory
267 vaccination campaign: no BT cases or laboratory positivity were registered from 2011 to date in
268 domestic animals, indicating that the virus was not re-introduced nor it overwintered in the
269 region. Also in seroprevalence studies, a decline in BTV seroprevalence in red deer was shown
270 after an epidemic event, or domestic livestock vaccination (Corbière et al., 2012; A. Linden et al.,
271 2010; Stallknecht et al., 1991), suggesting that red deer are not involved in the maintenance of
272 BTV for long periods (Falconi et al., 2011).

273 Our results confirm the serological positivities (3 out of 49 animals) observed in hunted red deer
274 from La Mandria in 2009-2010 (Radaelli et al., 2011); in this study low antibody titres were
275 recorded, and the seroneutralization test confirmed that the involved serotype was BTV-8.
276 Our work contributes to the knowledge on the role of wild ungulates in BTV-8 epidemiology in
277 north-western Italy, although further studies are needed to identify the factors that influenced the
278 virus dynamics in wildlife. Even though the role of wildlife as BTV reservoir in Piedmont is unlikely,
279 these animals serve as feeding source for culicoides and can be considered good sentinels of
280 infection (Falconi et al., 2011; Stallknecht and Howerth, 2004). Samples collection and testing,
281 indeed, is particularly easy in areas where wild ungulates are routinely subjected to population
282 control plans and hunted. Wild ungulates could thus be very useful for BTV surveillance in areas
283 with promiscuous presence of wild and domestic animals.
284
285 Conflict of interest
286 No competing financial interests exist.
287
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293
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372

373 Table 1. Prevalence of BTV infection in ungulates hunted in Piedmont in 2008-2011

Category		**No. tested**	**Prevalence of infection (n, 95% CI)**
Species			
	Red deer	102	20.6% (21, 13.2-29.7)
	Roe deer	78	15.4% (12, 8.2-25.3)
	Alpine chamois	55	7.3% (4, 2.0-17.6)
Hunting season			
	2008-2009	16	25% (4, 7.3-52.4)
	2009-2010	135	19.3% (26, 13-26.9)
	2010-2011	84	8.3% (7, 3.4-16.4)
Age			
	<1 year	59	16.9% (10, 8.4-29.0)
	>1 year	176	15.3% (27, 10.4-21.5)
Sex			
	Female	119	19.3% (23, 12.7-27.6)
	Male	116	12.1% (14, 6.8-19.4)
Province			
	Cuneo	128	14.8% (19, 9.2-22.2)
	Torino	107	16.8% (18, 10.2-25.3)

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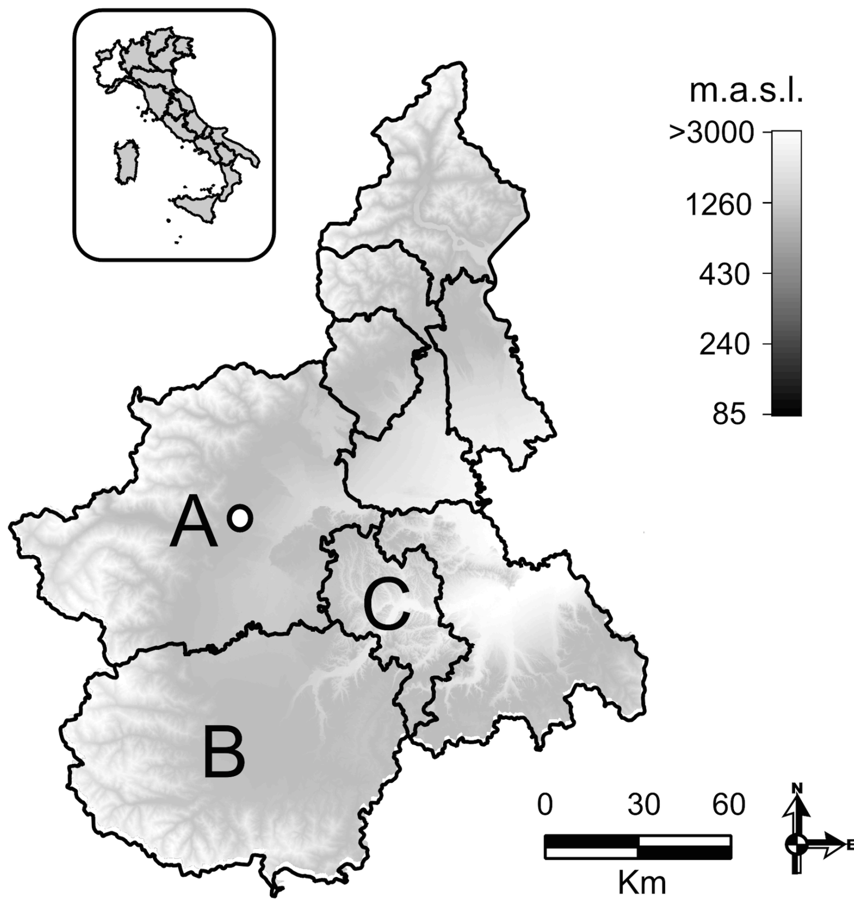

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382 Fig. 1. Map of Piedmont region with administrative provinces with BTV-8 outbreaks in 2008-2010

383 (A= Torino, B= Cuneo, C= Asti; circle= La Mandria park) and elevation (meters above sea level,

384 a.s.l.).

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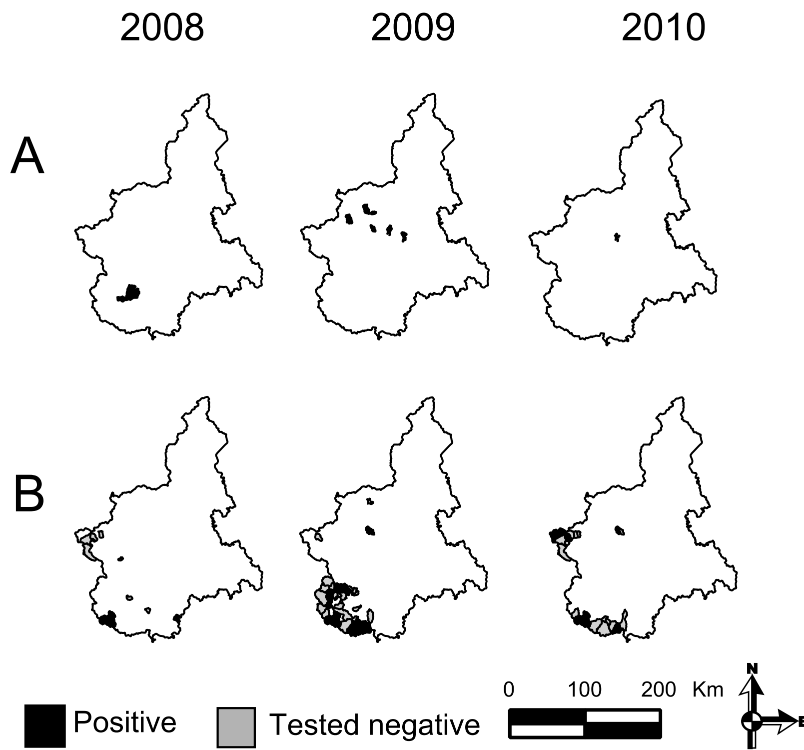

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387 Fig. 2. BTV-8 domestic outbreaks (A) and tested municipalities for BTV presence in wildlife (B;

388 gray= negative, black= positive for at least one tested animal) in Piedmont region, north-western

389 Italy, in 2008-2010.

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