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## Endemic papillomavirus infection of roe deer ()

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► **To cite this version:**

Károly Erdélyi, László Dencső, Róbert Lehoczki, Miklós Heltai, Krisztina Sonkoly, et al.. Endemic papillomavirus infection of roe deer (). *Veterinary Microbiology*, 2009, 138 (1-2), pp.20. 10.1016/j.vetmic.2009.02.002 . hal-00490542

**HAL Id: hal-00490542**

**<https://hal.science/hal-00490542>**

Submitted on 9 Jun 2010

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## Accepted Manuscript

Title: Endemic papillomavirus infection of roe deer  
(*Capreolus capreolus*)

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Solymosi



PII: S0378-1135(09)00080-7  
DOI: doi:10.1016/j.vetmic.2009.02.002  
Reference: VETMIC 4359

To appear in: *VETMIC*

Received date: 16-11-2008  
Revised date: 21-1-2009  
Accepted date: 3-2-2009

Please cite this article as: Erdélyi, K., Dencső, L., Lehoczki, R., Heltai, M., Sonkoly, K., Csányi, S., Solymosi, N., Endemic papillomavirus infection of roe deer (*Capreolus capreolus*), *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.02.002

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **Endemic papillomavirus infection of roe deer (*Capreolus capreolus*)**

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25

**26 Abstract**

27 Roe deer papillomavirus (CcPV1) infection has been identified as an endemic disease in roe  
28 deer populations of the Carpathian basin in Central Europe (Hungary, Austria and Croatia).  
29 The disease is characterized by easily recognizable skin tumours similar to Deer  
30 Papillomavirus infection of North American deer species. In 2006, a questionnaire study was  
31 conducted among all Hungarian game management units (GMUs) in order to assess the  
32 distribution of the disease and its major epidemiological features. Categorical information was  
33 collected about disease occurrence, trend and frequency of detection, on primarily affected  
34 age classes in both sexes, and association of lesions with mortality. Replies were received  
35 from 539 GMUs representing 50,9% of total GMU territory and disease presence was  
36 reported by 295 (54.7%) GMUs. Older age classes of both sexes were found to be more  
37 affected. Association of various environmental factors with disease occurrence was evaluated  
38 and data were collected on the occurrence of similar skin lesions in other European countries.  
39 Pathological features of CcPV1 infection were described and the localisation of both CcPV1  
40 antigen and DNA was characterised by immunohistochemistry and in-situ DNA hybridisation  
41 in skin lesions. Virus presence was also demonstrated by PCR and PCR product sequencing.

42

43 Keywords: papillomavirus, epidemiology, roe deer

44

45

**46 Introduction**

47 Papillomaviruses (PVs) cause neoplastic lesions in a wide range of mammalian and some bird  
48 species. PVs are epitheliotropic, non-enveloped, icosahedral viruses, 50-55nm in diameter,  
49 containing a circular, double-stranded DNA genome, ranging from 7.4 to 8.6 kb. Tumours  
50 induced by PVs are especially common in ruminants and humans. Papillomas,  
51 fibropapillomas or fibromas associated with PV infection have been reported in a number of  
52 cervid species e.g. white-tailed deer (Shope, 1958; Lancaster & Sundberg, 1982), mule deer

53 (Lancaster & Sundberg, 1982), European elk (Moreno-Lopez et al., 1981), reindeer (Moreno-  
54 Lopez et al., 1987), red deer (Moar & Jarrett, 1985; Sundberg, 1987; Erdélyi et al., in press),  
55 roe deer (Takács and Nagy-Bozsoki, 1998; Erdélyi et al., 2008). The complete genomes of six  
56 cervid papillomaviruses have been published so far (Groff and Lancaster, 1985; Ahola et al.,  
57 1986; Stenlund et al., 1983; Moreno-Lopez et al., 1987; Gottschling et al., 2008; Erdélyi et  
58 al., 2008). The best known and most widely distributed cervid PV causing skin and lung  
59 fibromatosis is the deer papillomavirus (DPV), infecting primarily White-tailed deer  
60 (*Odocoileus virginianus*) in North America (Shope, 1958; Sundberg and Lancaster, 1988).  
61 Presumably, the same virus also infects other closely related deer species i.e. Mule deer and  
62 Black-tailed deer (*Odocoileus hemionus*) inhabiting the same areas (Sundberg and Lancaster,  
63 1988). Another cervid PV with a wide distribution is the European elk papillomavirus  
64 (EPPV), inducing skin tumours (Borg, 1975) and pulmonary fibromatosis (Moreno-Lopez et  
65 al., 1986) of European elk (*Alces alces*) in Scandinavia. Epidemiological studies of DPV  
66 infections suggest that PVs spread in wild cervid populations by direct contact and blood-  
67 feeding insect vectors (Sundberg and Nielsen, 1981). The disease is assumed to be self  
68 limiting and DPV mainly affects younger animals. In contrast however, fibropapillomas are  
69 most commonly seen in older specimens of European elk and moose (Sundberg, 1987).  
70 Roe deer fibropapillomatosis (Kocsner, 2001; Takács and Nagy-Bozsoki, 1998) is an endemic  
71 viral disease producing typical skin tumours, similar to DPV infection of North American  
72 deer species and endemic fibromatosis of European elk in Sweden. The causative agent is a  
73 novel Roe-deer papillomavirus (CcPV1) belonging to the ruminant-specific Delta-  
74 papillomavirus genus (Erdélyi et al., 2008), affecting roe-deer populations of the Carpathian  
75 basin (Hungary, eastern parts of Austria (Theodora Steineck personal communication) and  
76 northern parts of Croatia (Salajpal, et al., 2006)).  
77 The disease had been first described in certain parts of Hungary during the early nineteen-  
78 sixties (Kocsner, 1996) but the first attempts to characterise the contemporary disease  
79 situation are fairly recent. Kocsner (2001) reported the presence of fibropapillomas in three

80 distinct foci and a 1,1% prevalence of the infection in one closely studied roe deer population.  
81 Takács and Nagy-Bozsoki (1998) identified a fourth location where they detected the disease  
82 in 1996. Histopathological and immunohistochemical studies of roe deer fibropapillomas  
83 demonstrated the presence of papillomavirus antigen (Kocsner, 2001) and a complete genome  
84 of CcPV1 has also been identified in these same lesions (Erdélyi et al., 2008).

85 The aim of this study was to assess the epidemiological and pathological features of roe deer  
86 fibropapillomatosis, its distribution area within Hungary and its status in a wider European  
87 context.

88

## 89 **Material and methods**

### 90 *Questionnaire survey*

91 A mailed questionnaire survey was conducted among all Hungarian game management units  
92 (GMUs) i.e. local Hunting Associations in order to assess the distribution and ecology of the  
93 disease. Questionnaires were mailed at the end of March 2006 to all 1201 GMUs then in  
94 existence, covering the whole territory of Hungary. The one page multiple choice  
95 questionnaire was accompanied by a single page covering letter explaining the purpose of the  
96 study and containing photographic illustrations of the lesions typical for the disease. Replies  
97 were signed by presidents of hunting associations thus authorizing data use, but the actual  
98 disease information was most often provided by professional hunters managing the hunting  
99 areas. A reminder was sent out to non-responding GMUs two weeks after the response  
100 deadline. Knowing that comprehensive statistics or records of disease outbreaks, mortality  
101 data or diagnoses are not available at the vast majority of GMUs the questionnaire was  
102 designed to collect categorical information about disease occurrence, trend and frequency of  
103 detection, on primarily affected age classes in both sexes, and the association of lesions with  
104 roe deer mortality.

105 Disease occurrence was categorized as: 1. Not detected; 2. Intermittent; and 3. Permanent. A  
106 question on how many cases were detected over the past five years could be answered by one

107 of three categories: 1. An odd case over several years; 2, 1-2 cases per year; and 3. Several  
108 cases per year. These categories were coded into “Disease Frequency Indicator” values (DFI  
109 1-3) which were later used to characterise the spatial distribution and the intensity of the  
110 infection.

111 The trend of disease occurrence could be characterized as 1. Declining; 2. Stagnating; or 3.  
112 Increasing. Identification of primarily affected age classes of both sexes (juveniles, subadults  
113 and adults) was also requested. The adult male category was further divided into middle aged  
114 and old bucks, as these age classes can be safely distinguished by visual observation methods.  
115 The question whether the described lesions were seen on animals found dead at the GMU was  
116 to be answered by Yes or No.

117 Additional data were collected on the occurrence of roe deer fibropapillomas throughout  
118 Europe, from institutions performing wildlife disease diagnostics or maintaining wildlife  
119 disease databases. Available databases were searched retrospectively for diagnoses of skin  
120 tumours in general and specifically papillomas, fibropapillomas or fibromas found in roe deer  
121 and for the total number of roe deer samples submitted for examination during the same time  
122 period. Institutions kindly providing these data were: AFSSA-LERRPAS (SAGIR database)  
123 France, Institute for Zoo and Wildlife Research (IZW) Berlin, Germany, National Veterinary  
124 Institute (SVA) Uppsala Sweden, Veterinary Laboratories Agency Diseases of Wildlife  
125 Scheme (VLADoWS), United Kingdom.

126

### 127 *Data and analysis*

128 Roe deer population data (yearly population size estimates and hunting bag sizes for bucks,  
129 does and fawns) were obtained from the Hungarian National Game Management Database  
130 (OVA). Averages of the previous six years’ data (2001-2006) were calculated for each GMU  
131 separately and used in the study. Retrospective roe deer population data were obtained for the  
132 whole of France from Réseau Ongulés Sauvages - ONCFS-FNC-FDC. Spatial analysis and  
133 visualisation of data was performed in ArcGIS 9.0 (Environmental Systems Research

134 Institute). Data of individual environmental categories were extracted from the Corine Land  
135 Cover 2000 database (CLC2000) (European Environment Agency) for every GMU territory  
136 and used for subsequent analyses. R-environment was used for statistical analysis and result  
137 visualisation (R Development Core Team, 2008).

138 Using the GMUs with known disease status and the proportion of CLC2000 classes within  
139 these GMUs a classifier was created based on random forest supervised machine learning  
140 algorithm (Breiman, 2001). This algorithm was used to predict the disease status of non-  
141 responding GMUs and estimate the wider distribution area of the disease based on population  
142 density and the CLC2000 environmental data. Logistic regression and Pearson's product  
143 moment correlation coefficient was used to quantify the association of disease occurrence,  
144 environmental and host population data.

145

#### 146 *Sample collection*

147 All 42 roe deer carcasses submitted for post mortem examination to the Central Veterinary  
148 Institute in the period between 2004 and 2006 from different parts of Hungary were examined  
149 for the presence of skin lesions. Tissue samples were collected in 10% neutral buffered  
150 formalin for histopathology and frozen at -20°C for subsequent DNA extraction. Formalin  
151 fixed tissues were embedded in paraffin and cut into 4 µm thick sections. Three sections from  
152 each block were mounted on 3-aminopropyltriethoxy-silane (Sigma Aldrich Co., St Louis,  
153 MO, USA) treated glass slides and submitted for Hematoxylin-Eosin (HE) staining,  
154 immunohistochemistry (IHC) and in-situ hybridisation (ISH).

155

#### 156 *Virus identification and visualisation*

157 PCR assays were performed to demonstrate the presence of CcPV1 DNA in fibropapillomas  
158 of roe deer originating from distinct locations in Hungary as described earlier by Erdélyi et al.  
159 (2008). Amplified PCR products were directly sequenced from both directions with the



160 primers used for amplification (Pap1f and Pap1r) on an ABI 373A automated DNA sequencer  
161 (Applied Biosystems, Foster City, CA, USA).

162 Viral antigen was visualised in tumour tissues by the immunohistochemistry method  
163 described by Erdelyi et al. (in press). Briefly, after antigen retrieval (0.1% protease XIV  
164 solution (Sigma Aldrich Co., St Louis, MO, USA) at 37 °C for 10 min) sections were first  
165 incubated in 3% H<sub>2</sub>O<sub>2</sub> solution for 10 min and then in a 2% solution of skimmed milk  
166 powder for 20 min. Polyclonal rabbit anti BPV1 antibodies (B0580, DAKO, Glostrup,  
167 Denmark) targeting the BPV-1 L1 capsid protein were added to the sections at a 40000 fold  
168 dilution and incubated at 4 °C overnight. Antibody binding was detected by horseradish  
169 peroxidase-labelled polymer (EnVision™+ Kit; Dako, Glostrup, Denmark).

170 In-situ DNA hybridisation was performed with a 392 bp long DIG labelled DNA probe  
171 synthesised by PCR from the L1 ORF region of the CcPV1 genome (nt 6407-6798;  
172 EF680235), using the PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Mannheim,  
173 Germany) according to the manufacturer's instructions. The original Pap2 fragment used for  
174 sequencing of the CcPV1 genome (Erdélyi et al., 2008) served as a template for probe  
175 synthesis. The UPV1 (5'-HATWSARGAYGGDGANATG-3') and UPV2 (5'-  
176 CCRTTRTTHHDDCCYTG-3') primer pair utilised in this reaction was originally designed  
177 for the detection of all Delta PVs with PRIMER2 (Scientific and Educational Software,  
178 Stateline, PA, USA) and PrimerSelect from the Lasergene software package (DNASTAR  
179 Inc., Madison, WI, USA). The hybridisation reaction was performed according to the "In-situ  
180 hybridisation to DNA in cells and tissue sections" protocol by Schwarzacher and Harrison  
181 (2000). Briefly, the mounted sections were dewaxed and rehydrated according to routine  
182 procedures and treated with proteinase K (20 µg/ml) at 37°C for 30 minutes followed by a 4%  
183 paraformaldehyde fixation at 4°C for 20 minutes. All slides were incubated with 200 µl  
184 aliquots of hybridisation buffer (50% formamide; 2x standard saline citrate buffer (SSC); 1  
185 mM Tris HCl, pH 7; 0.1 mM EDTA; 30 ng/µl ssDNA; 10% dextrane sulphate) for 2 hours at  
186 42°C and thereafter replaced by 60 µl of hybridisation mix containing 50 ng/µl of DIG

187 labelled DNA probe diluted in hybridisation buffer. Target DNA was denatured at 95°C for  
188 10 minutes, then gradually cooled down to 42°C and allowed to hybridise overnight (20  
189 hours) in a moist chamber. Sections were rinsed in 2x SSC solution and then gently shaken in  
190 a posthybridisation stringent wash solution (0.1x SSC; 2 mM MgCl<sub>2</sub>; 0.1% Tween 20) for 45  
191 minutes at 42°C. Slides were finally washed twice in 1x SSC for 10 minutes at room  
192 temperature and once in 1x PBS for 5 minutes. The hybridisation reaction was visualized  
193 using the DIG Nucleic Acid Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany)  
194 according to the manufacturer's instructions and counterstained with methyl green.

195

## 196 **Results**

### 197 *Pathology and virus demonstration*

198 From the 42 roe deer carcasses examined between 2004 and 2006, 14 showed macroscopic  
199 lesions consistent with skin fibropapillomatosis. Fibropapillomas of roe deer were found to be  
200 firm, round, multiple skin tumours of varying size. On average the lesions were 3-5 cm in  
201 diameter (Figure 1.) but sometimes they reached 11-15 cm. Although the number of  
202 fibropapillomas on one animal was usually less than 10, individuals carrying more than 150  
203 tumours were also found. Fibropapillomas were typically localised on the head, neck, belly  
204 and legs of infected animals. Some tumour surfaces exhibited moderate and transient  
205 papillary structure but the typical appearance was of a firm, hairless mass covered with  
206 smooth or verrucous epidermis. The epidermis was often pigmented and the surface of larger  
207 tumours was frequently eroded and ulcerated. The main tumour mass was located in the  
208 dermis and microscopically it consisted of groups of proliferating fibroblasts embedded in a  
209 compact mesh of collagen fibres, usually covered by a layer of loosely arranged collagen  
210 fibres and fibroblasts of the upper dermis. The epidermis showed signs of acanthosis and  
211 hyperkeratosis (Figure 2.). Occasional groups of keratinocytes containing moderate amounts  
212 of dark brown, coarse melanin granula were seen in the stratum spinosum and variable  
213 quantities of basophilic, keratohyalin granula were present in cells of the stratum granulosum.

214 PV DNA was successfully amplified by PCR from samples of all eight selected cases  
215 exhibiting typical skin lesions. All obtained nucleotide sequences were found to be identical.  
216 Both PV L1 antigen and DNA were visualized only in the epidermis covering the tumour  
217 stroma. PV DNA homologous to the L1 ORF sequence was present in a few keratinocyte  
218 nuclei of the stratum basale but the stratum spinosum and stratum granulosum had much  
219 larger numbers of keratinocytes containing PV L1 DNA in both their nuclei and cytoplasm  
220 (Figure 3a). PV major capsid antigen was predominantly localised in the cytoplasm and nuclei  
221 of keratinocytes of the stratum granulosum (Figure 3b), but antigen aggregations were often  
222 observed between the keratin layers of the stratum corneum as well.  
223 The nodular lung fibrosis occasionally associated with DPV infection was not detected in roe  
224 deer with skin fibropapillomatosis. However, the presence of a moderate, diffuse lung fibrosis  
225 was seen in two completely processed cases, but neither L1 antigen nor L1 DNA could be  
226 visualised in these lesions by IHC and ISH respectively.

227

## 228 *Epidemiology*

229 Replies to the questionnaire were received from 539 GMUs. The overall questionnaire  
230 retrieval rate was 45,3% representing 50,9% of the total territory of all Hungarian GMUs i.e.  
231 effectively half of the country. The occurrence of the disease was reported by 293 GMUs  
232 (54,4% of responders), characterised as intermittent occurrence by 200 (37,1%) and  
233 permanent presence by 93 (17,4%) GMUs. Regarding frequency of disease detection, an odd  
234 case over several years (DFI=1) was reported by 126 GMUs (43% of positive territories), 1-2  
235 cases per year (DFI=2) by 109 (37,2%) and more than 2 cases per year (DFI=3) by 58  
236 (19,8%). Association plots of disease presence versus DFI categories (Figure 4.) showed an  
237 overlapping, positive and significant association ( $p<0.001$ ) between permanent disease  
238 presence and DFI categories 2 and 3, and between intermittent disease presence and DFI  
239 categories 1 and 2. The trend of disease occurrence was characterized as declining in 60  
240 (21,1%), stagnating in 187 (65,6%) and increasing in 38 (13,3%) GMUs.

241 Bucks were found to be only slightly more affected (51,2%) by the disease than does (48,4%)  
242 but this difference was not statistically significant (McNemar's Chi-squared test with  
243 continuity correction,  $p=0.7657$ ). The distribution of primarily affected age classes within  
244 each sex (bucks/does) was 2,3%/2,8% juveniles, 14,9%/35,8% subadults, 56,6% middle aged  
245 adult bucks, 26,2% old adult bucks and 61,4% adult does (Figure 5.). Differences detected in  
246 the age distribution of the disease within each sex were found to be significant by Pearson's  
247 Chi-squared test ( $p<0.001$ ). Fibropapillomas were seen on animals found dead in 34 GMUs,  
248 i.e. 11% of the 309 units answering this question.

249 Observed (questionnaire data) and predicted (for non-responding GMUs) spatial distribution  
250 of the disease is presented on Figure 6, characterised by DFI values. Both random forest  
251 algorithms produced for disease occurrence and frequency data utilized the following  
252 variables: Non-irrigated arable land (CLC-code 211), Pastures (CLC-code 231), Broad-leaved  
253 forest (CLC-code 311), Water courses (CLC-code 511) and Roe deer density.

254 As the preliminary visual assessment of disease distribution and random forest variable  
255 importance values suggested a potential effect of lowland areas and major waterways on PV  
256 distribution, we tested the association of disease occurrence with water courses, forested areas  
257 and roe deer population density data. Results of the logistic regression analysis showed that  
258 both roe deer density (OR: 1.284, 95% CI: 1.151-1.433,  $p<0.001$ ) and the presence of  
259 waterways (OR: 1.318, 95% CI: 1.105-1.572,  $p=0.002$ ) increased, while broad-leaved forest  
260 cover decreased (OR: 0.974, 95% CI: 0.965-0.984,  $p<0.001$ ) the likelihood of infection by  
261 CcPV1 (Figure 7.). Pearson's product moment correlation coefficient analysis showed no  
262 association ( $R: 0.07$ ,  $p=0.12$ ) between water courses and roe deer density, a weak negative ( $R:$   
263  $-0.244$ ,  $p<0.001$ ) association between broad-leaved forest cover and roe deer density, and a  
264 moderate, positive association between non-irrigated arable land cover and roe deer density  
265 ( $R: 0.303$ ,  $p<0.001$ ).

266 An extensive literature search and the results of a survey of European wildlife disease  
267 databases showed that the endemic presence of roe deer fibropapillomatosis has not been

268 detected by the specialist laboratories in France, Sweden, Germany or United Kingdom. A  
269 total of 10585 roe deer carcasses were examined in France during the 1986-2007 period with  
270 only a single case of a skin tumour, a sarcoid-like lesion detected on the eyelid of a roe deer  
271 doe found dead in 2005. In a review of 4168 roe deer cases processed at SVA in the 1947-  
272 1982 period, there is no mention of roe deer fibropapillomas amongst the diagnosed  
273 neoplastic diseases (Borg, 1991). However, a photograph of a fibroma-like solitary tumour on  
274 the head of a roe deer is presented in an earlier review (Borg, 1970). Additionally, a single  
275 case of a solitary skin fibroma and a single fibrosarcoma had been diagnosed in the 2417 roe  
276 deer samples submitted to SVA between 1986 and May 2008. No skin tumours were detected  
277 on any of the 231 roe deer examined between 1972 and 2008 at the pathology unit of the  
278 IZW, Berlin, Germany. According to the VLA Diseases of Wildlife Scheme (VLADoWS)  
279 database no skin tumours were found on the 70 roe deer carcasses examined between 2000  
280 and 2008 in the United Kingdom.

281

## 282 Discussion

283 Pathomorphological and epidemiological features of CcPV1 infection closely resemble DPV  
284 infections of white-tailed deer and other indigenous deer species in North America, except for  
285 the absence of nodular lung fibromatosis. The possible implication of CcPV1 in the mild  
286 fibrotic lung lesions of roe deer could be examined further by in-situ demonstration of the  
287 activity of early PV genes and proteins. However, the finding that the disease predominantly  
288 affects older age classes of both sexes is similar to the epidemiology of fibropapillomatosis in  
289 European elk and Moose in contrast to DPV induced lesions which are more common in  
290 young white-tailed deer. This feature also suggests a cumulative disease tendency and a slow  
291 regression of lesions, but our data are not sufficient to estimate the duration of disease.

292 Results of the questionnaire survey confirmed the presence of roe deer fibropapillomatosis in  
293 areas identified by all our earlier case records and with the data presented by Kocsner (1996;  
294 2001) and Takács and Nagy-Bozsoki (1998). Many infected areas were identified for the first

295 time and were confirmed by additional samples submitted after the survey. The positive  
296 statistical correlation of disease distribution with both host density and waterways, and the  
297 fact that there was no correlation between these two variables suggests that waterways may  
298 have an independent effect on the distribution of roe deer fibropapillomatosis. One of the  
299 possible explanations, which should be experimentally tested, is that the habitats in question  
300 may be especially suitable for arthropod vectors which might play a significant role in the  
301 ecology of CcPV1. However, the potential effect of an increased, seasonal roe deer  
302 aggregation induced by these habitats in an agricultural landscape should not be excluded  
303 either.

304 The endemic, geographically confined distribution of roe deer fibropapillomatosis established  
305 by our study lends further support to the hypothesis that host related genetic factors may have  
306 an influence on the susceptibility of roe deer populations to CcPV1 infection (Erdélyi et al.,  
307 2008). The emergence of an introduced CcPV1 infection would have been expected in other  
308 susceptible roe deer populations, especially considering the high population densities  
309 throughout Europe (e.g. France: 9,503 km<sup>-2</sup>, Hungary: 3,404 km<sup>-2</sup>), the universal presence of  
310 other environmental stress factors and the multiple translocation events from endemic areas in  
311 the past (Vernesi et al., 2002).

312 Despite its practicality, the drawback of our survey approach is that classical epidemiological  
313 indicators (e.g. disease prevalence or incidence) cannot be calculated from the categorical  
314 data provided, and that certain bias may be inherent in the estimates provided by interviewees.  
315 This may be most pronounced in the answers provided by those GMUs where disease  
316 occurrence is scarce.

317 Nevertheless, results of this study indicate that there is a certain niche for questionnaire  
318 surveys in the study of wildlife disease ecology. Although skin tumours are special because of  
319 their more memorable and easily recognizable physical aspect, we believe that territorially  
320 organised game management and nature conservation systems may be used to obtain baseline  
321 data on the occurrence, distribution and the estimated or perceived impact and importance of

322 wildlife diseases. Results of such initial enquiries could be used to establish targeted research  
323 and surveillance of specific pathogens or host populations.

324

### 325 **Acknowledgements**

326 This research was partly funded by the Game Management Fund grant No 222/2006/PO of  
327 the Ministry of Agriculture and Rural Development and the National Scientific Research  
328 Foundation grant OTKA T049157. We would like to thank Marie-Eve Terrier (AFFSA,  
329 SAGIR database, France), Olivier Alabaric (Ecole Nationale Vétérinaire de Nantes, France),  
330 Erik Agren (SVA, Uppsala, Sweden), Gudrun Wibbelt (IZW, Berlin Germany), Paul Duff  
331 (VLADoWS, United Kingdom) for contributing the search results of their databases and  
332 Christine Saint-Andrieux (ONCFS, France) for providing French roe deer population data.

333

334

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401

402 **Figures**

403 **Figure 1.** Typical appearance of fibropapillomas on the head and hind legs of a roe deer buck.

404

405 **Figure 2.** Histological picture of a tumour surface exhibiting typical morphological features  
406 of a fibropapilloma with pronounced rete ridges formed by proliferating fibroblasts  
407 and collagen fibres, covered by the upper dermis and an epidermis showing signs of  
408 acanthosis and hyperkeratosis. Hematoxylin-Eosin, Bar = 50  $\mu$ m.

409

410 **Figure 3. A)** Immunohistochemistry visualizing aggregations of CcPV1 major capsid antigen  
411 in the cytoplasm and nuclei of keratinocytes of the stratum granulosum and between  
412 the keratin layers of the stratum corneum. **B)** In-situ hybridization showing the  
413 presence of Papillomavirus L1 ORF DNA in stratum spinosum and stratum  
414 granulosum keratinocyte nuclei and cytoplasm and fewer positive keratinocyte nuclei  
415 in the stratum basale. CcPV1 L1 ISH and anti BPV1 IHC. Bar = 100  $\mu$ m.

416

417 **Figure 4.** Association plot showing Pearson residuals of disease presence against disease  
418 frequency (DFI categories) revealing an overlapping, positive and significant  
419 association ( $p < 0.001$ ) between permanent disease presence and higher disease  
420 frequency (DFI categories 2 and 3) as well as between intermittent disease presence  
421 and low disease frequency (DFI categories 1 and 2).

422

423 **Figure 5.** Diagram of the distribution of primarily affected age classes within each sex  
424 (bucks/does).

425

426 **Figure 6.** Spatial distribution of roe deer fibropapillomatosis occurrence frequency among  
427 Game Management Units (GMUs) represented by DFI values (1-3) obtained from  
428 questionnaire data (Observed) and a Random forest based prediction for non-  
429 responding GMUs (Predicted). DFI values: 0 – No disease; 1 - An odd case over  
430 several years; 2 – One to two cases per year; 3 – More than 2 cases per year. Empty  
431 polygons – No response.

432

433 **Figure 7.** Results of the logistic regression analysis showing that both roe deer density (A)  
434 (OR: 1.284, 95% CI: 1.151-1.433,  $p < 0.001$ ) and the presence of waterways (B) (OR:  
435 1.318, 95% CI: 1.105-1.572,  $p = 0.002$ ) increase, while broad-leaved forest cover  
436 decrease (C) (OR: 0.974, 95% CI: 0.965-0.984,  $p < 0.001$ ) the odds of infection by  
437 CcPV1.

Figure 1





Figure 2

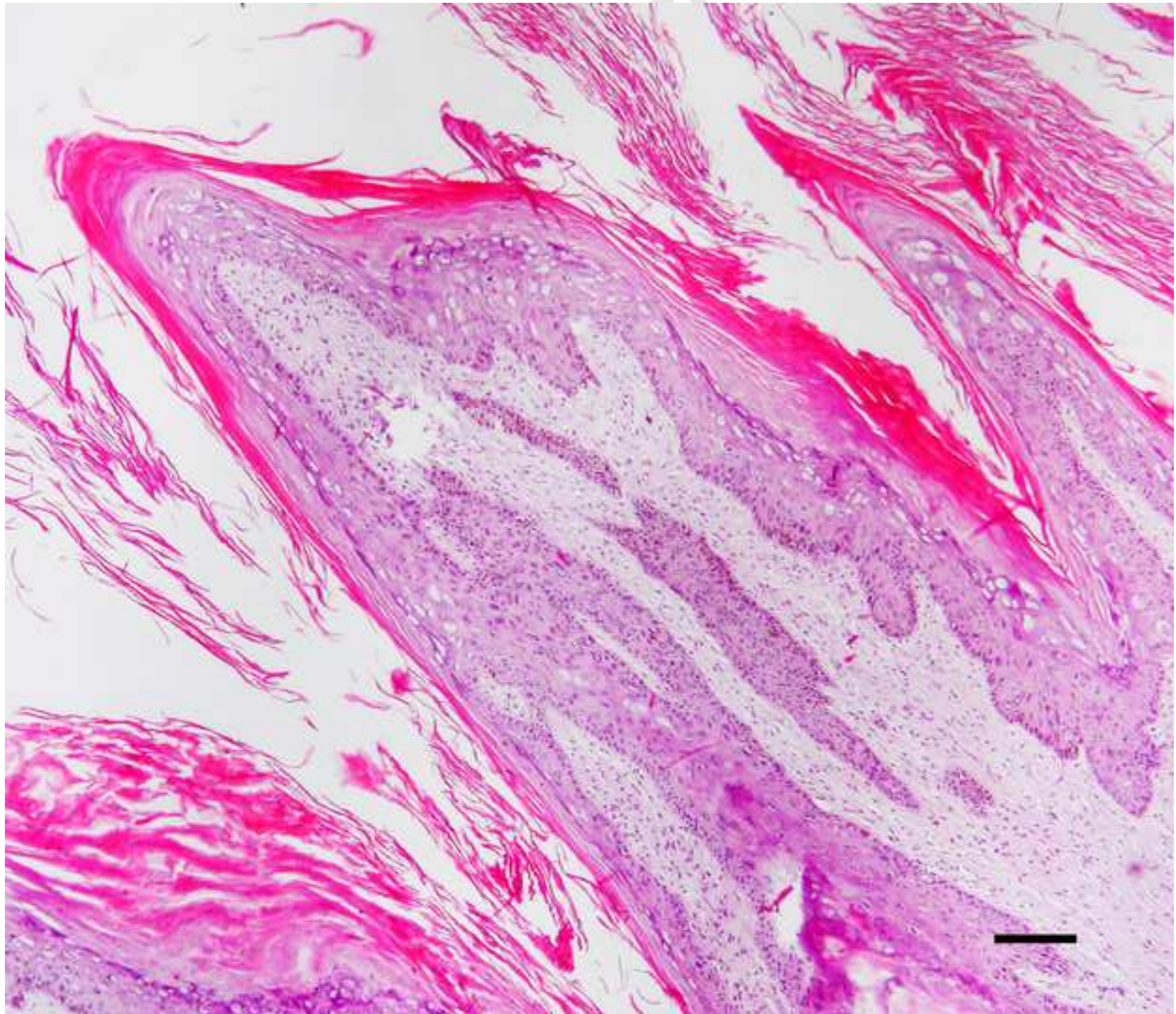




Figure 3

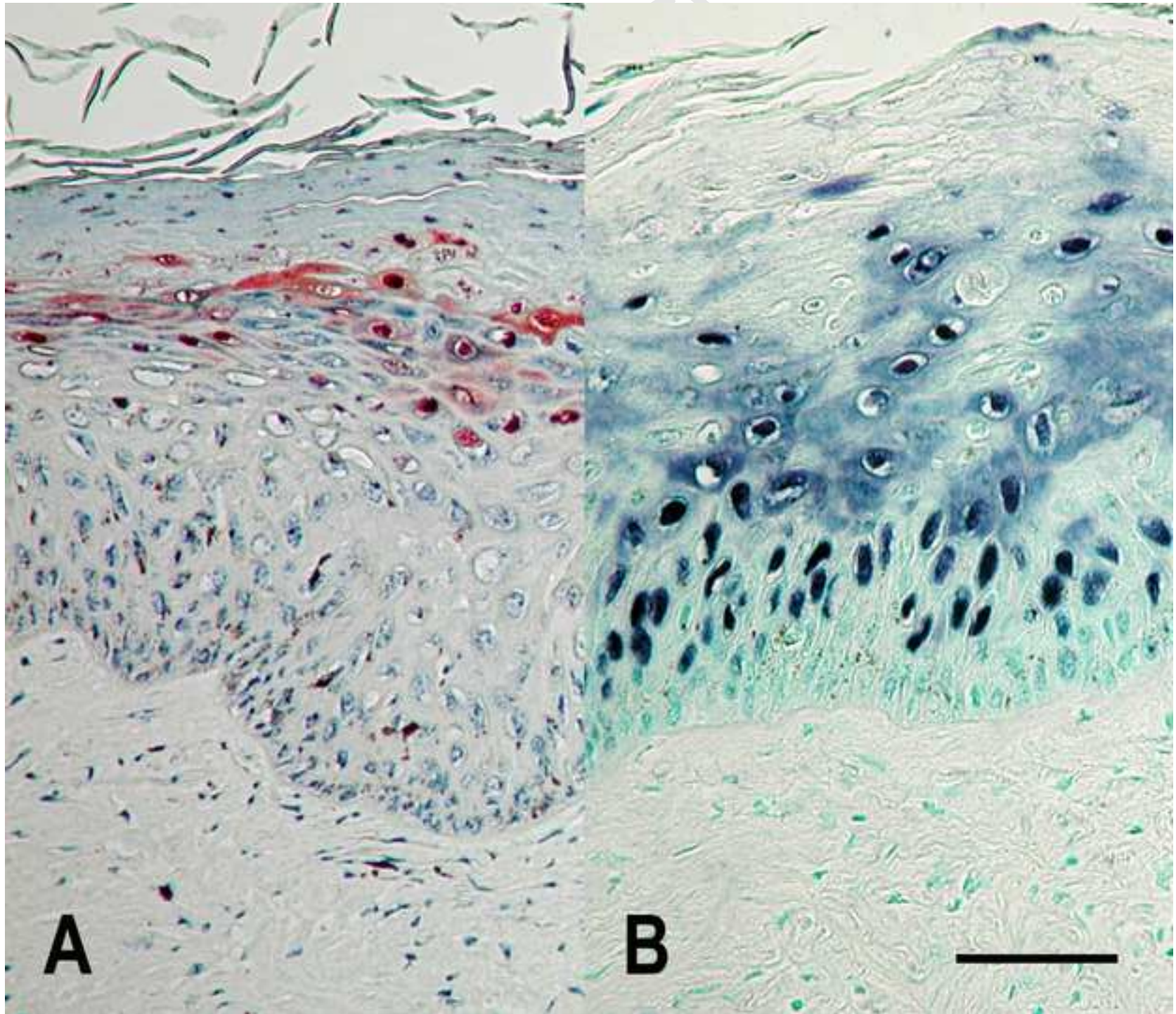


Figure 4

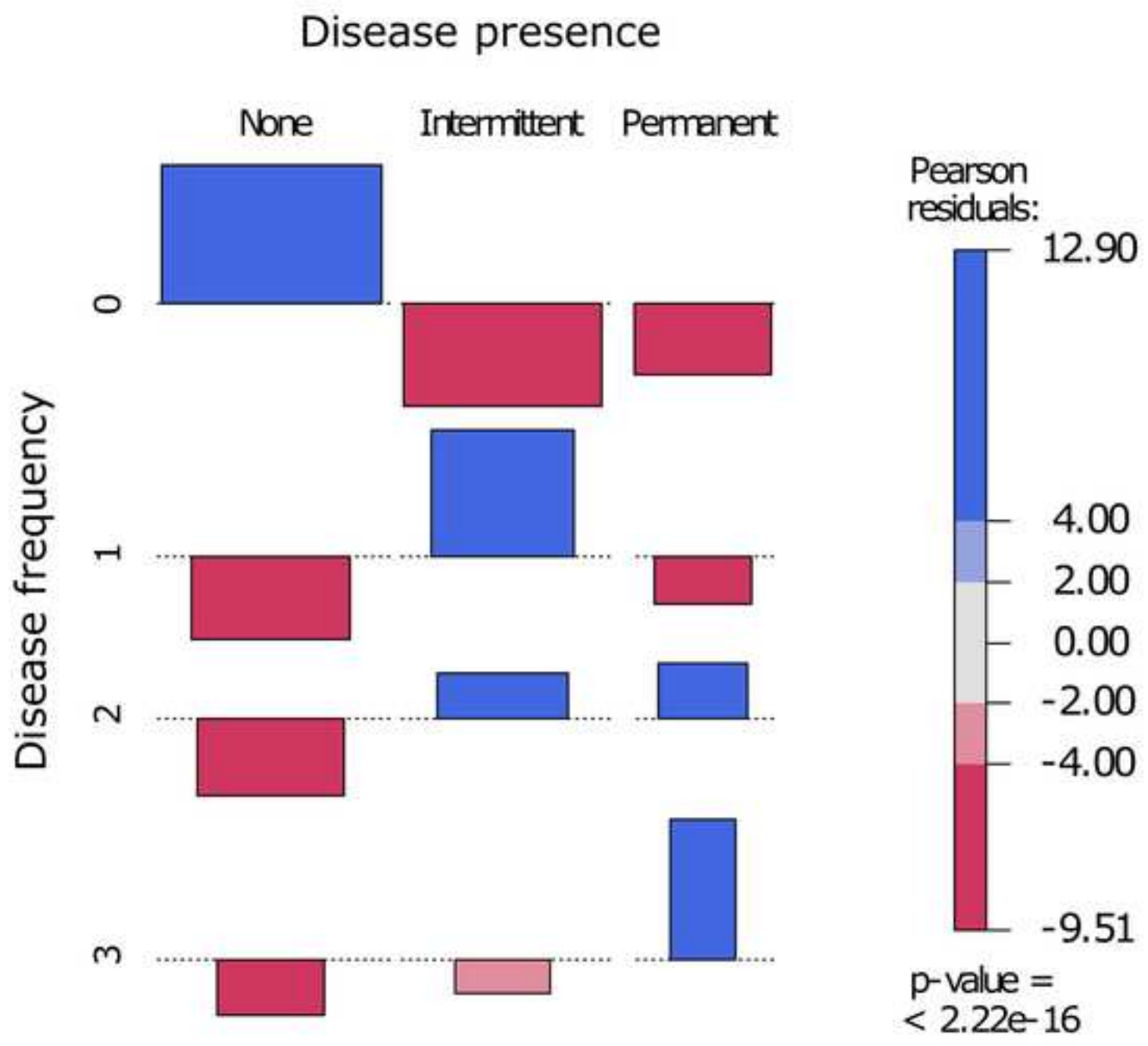
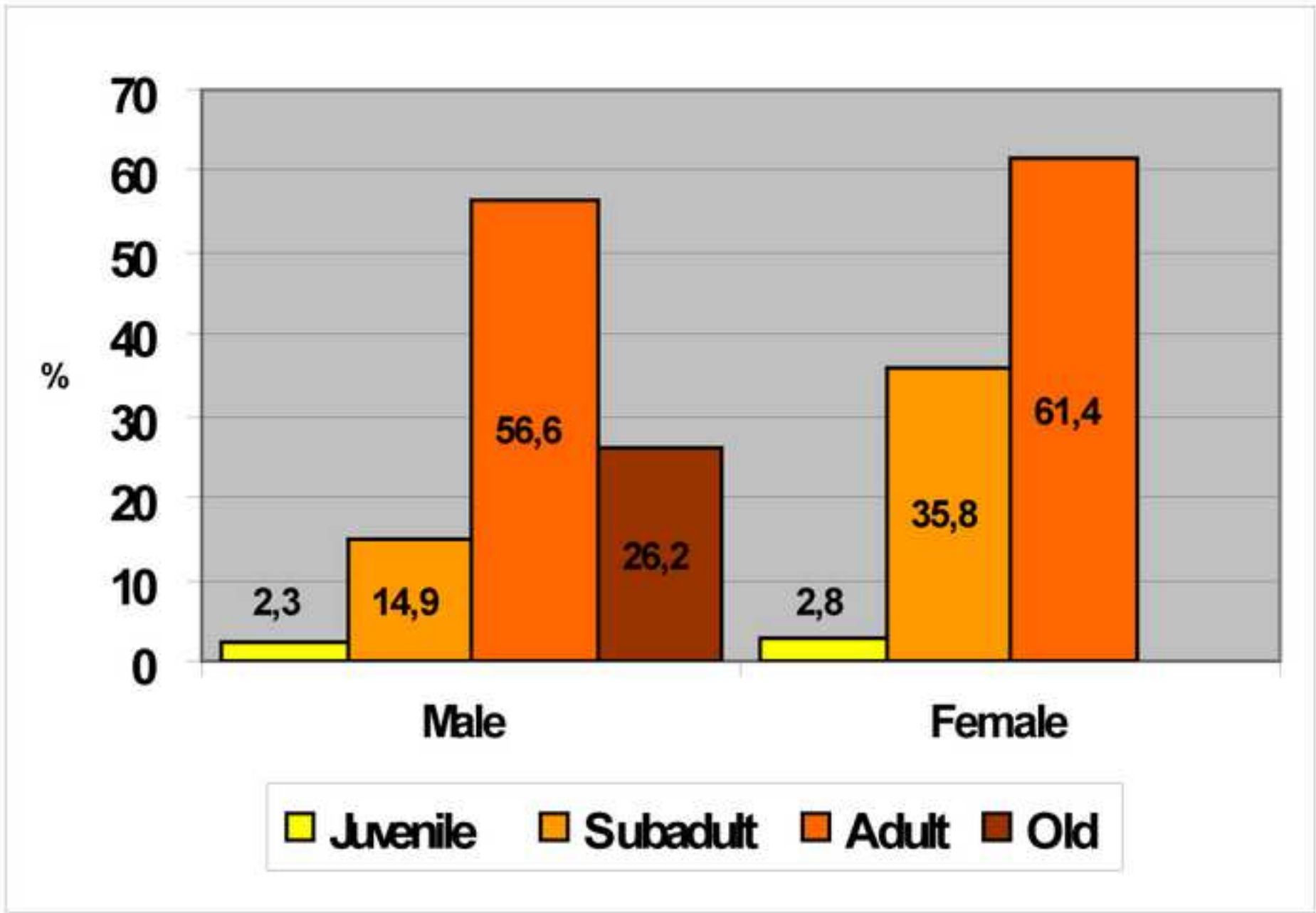
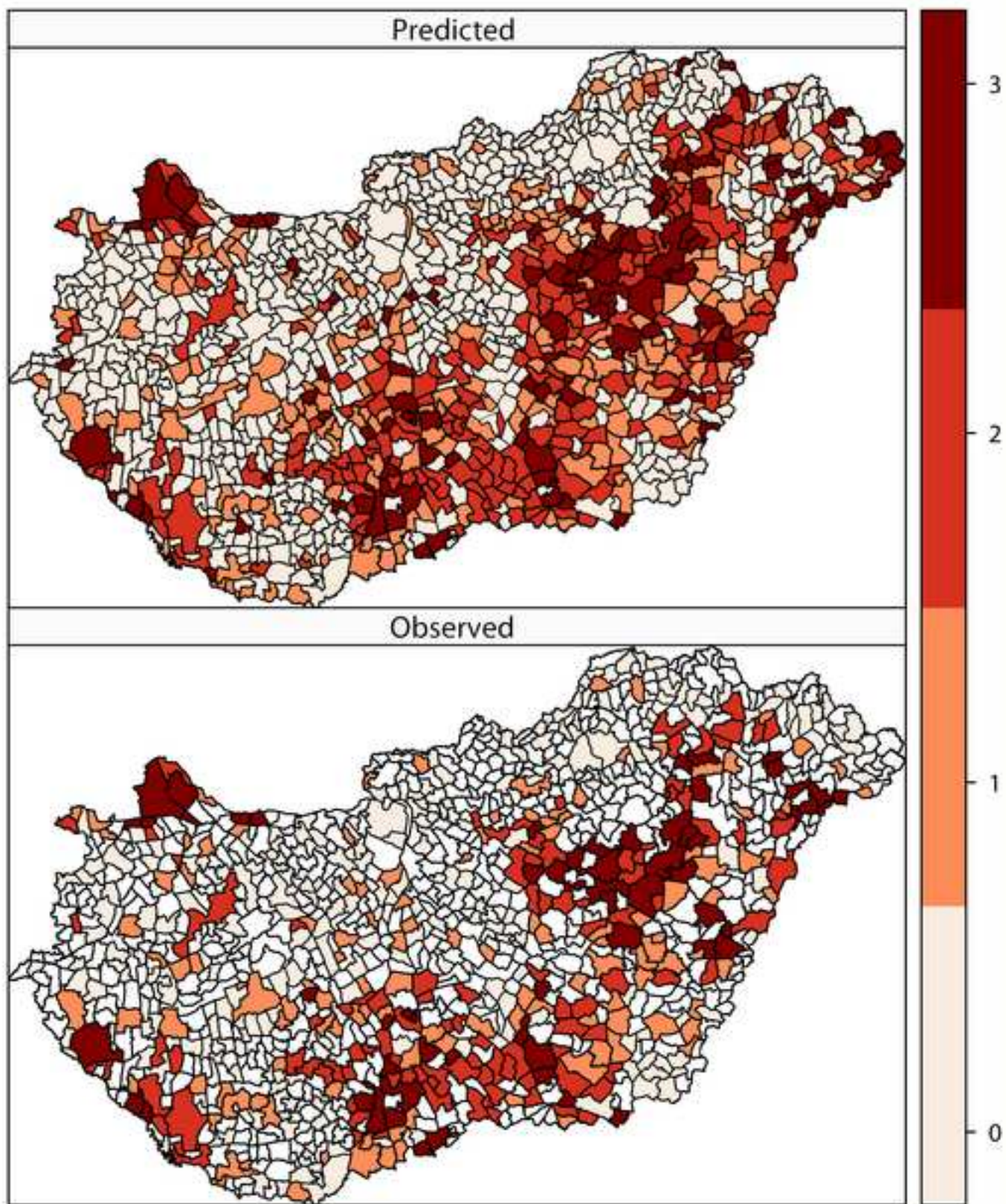


Figure 5

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