

1 **Temporal and spatial spread of *Hypoderma actaeon* infection in roe deer from**
2 **peninsular Spain determined by indirect ELISA**

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9

10 **Running head: Spread of *H. actaeon* infection in roe deer**

11

12 ABSTRACT

13 The host switching of *Hypoderma actaeon* (Diptera: Oestridae), an specific parasite of
14 red deer (*Cervus elaphus*), towards roe deer (*Capreolus capreolus*) has been recently
15 reported in Spain. In order to provide information about the temporal and spatial
16 spreading of *H. actaeon* infection in roe deer, 244 serum samples from animals hunted
17 in Spain between 2013 and 2018 were analyzed by indirect ELISA.

18 The overall seropositivity was 13.9%. Seropositivity was higher in continental (27.7%)
19 and mountainous (12%) areas from central Spain, followed by southern-mediterranean
20 (11.2%) and northern-oceanic regions (3.5%); differences were significant between
21 central-continental and northern-oceanic regions ($P=0.003$). No differences were found
22 according to the sex and age of roe deer ($P>0.05$). In 2013, all seropositive animals
23 were concentrated in two distant areas in central and southern of Spain, suggesting that
24 the host switch could have occurred independently in both regions. Changes in the
25 pattern of distribution of red deer and roe deer could have favoured the spreading of this
26 myiasis towards roe deer, indicating that roe deer may become infested by *H. actaeon* in
27 areas where both cervids coexist at high densities.

28

29 *Key words:* *Hypoderma actaeon*, antibodies, Iberian peninsula, indirect ELISA,
30 Oestridae, roe deer

31

32 INTRODUCTION

33 Warble flies are restricted to the Holarctic region where they are considered host-
34 specific of various Bovidae and Cervidae (Colwell *et al.*, 2006).

35 The species affecting Cervidae include *Hypoderma diana* and *Hypoderma actaeon*.
36 *Hypoderma diana* is mainly reported in central and eastern Europe and Asia (30° to 60°
37 North), with very sporadic reports in southern Europe (Reina *et al.*, 1992). This species
38 exhibits an exceptional adaptability to different species of Cervidae; thus, in addition to
39 the European roe deer (*Capreolus capreolus*) which is considered its main host, it was
40 also identified in red deer (*Cervus elaphus*), fallow deer (*Dama dama*), Eurasian elk
41 (*Alces alces*), Dybowski sika deer (*Cervus nippon dybowskyi*) (Pavlásek & Minář,
42 2014) and reindeer (*Rangifer tarandus*) (Kettle & Utsi, 1955). Although rarely, it has
43 been also detected infesting some Bovidae, such as the chamois (*Rupricapra*
44 *rupricapra*) and the mouflon (*Ovis musimon*) (Minář, 1980), and even other orders of
45 ungulates as wild boars (*Sus scrofa*) (Pavlásek & Minář, 2014) and horses (Kudrnáčová
46 *et al.*, 2014; Borges *et al.*, 2019).

47 In contrast with *H. diana*, the myiasis caused by *H. actaeon* is considered strongly
48 specific of red deer, with only sporadic reports in fallow deer (Panadero *et al.*, 2010)
49 and cattle (Ahmed *et al.*, 2017). The presence of this species is restricted to Europe
50 (Zumpt, 1965).

51 Studies developed in the last decades demonstrated that warble fly (WF) infestations are
52 very common in red deer from Spain, with prevalence ranging from 44.8% (de la Fuente
53 *et al.*, 2001) to 92% (Pérez *et al.*, 1995), being *H. actaeon* the single identified species
54 (San Miguel *et al.*, 2001; Panadero *et al.*, 2010). However, WF infestations in roe deer,
55 the most abundant Cervidae in the northern third of the Iberian Peninsula, are restricted
56 to a single report by *H. diana* in 1992 (Reina *et al.*, 1992). In 2016, Panadero *et al.*
57 (2016) reported for the first time infestation by larvae of *H. actaeon* in roe deer from
58 central Spain. Since that, several infestations by this species were registered in the same
59 area with a considerable number of larvae (Panadero *et al.*, 2018).

60 The main drawback for WF control in wildlife is the difficulty of performing an
61 accurate diagnosis, since samplings are mainly restricted to hunting periods, which
62 often do not overlap with the most suitable time for warble detection. In these
63 conditions, warble examination would underestimate the level of infestation; therefore,
64 immunological methods, based on the detection of specific antibodies, constitute a
65 useful tool to determine the exposure of animals to WF (Panadero *et al.*, 2010). The use

66 of larval antigens obtained from first instars of *H. lineatum* collected in slaughtered
67 cattle has demonstrated to be a suitable option for the diagnosis of *Hypoderma* spp.
68 infestations in Cervidae (Panadero *et al.*, 2010; Domínguez *et al.*, 2010). In this sense,
69 Panadero *et al.* (2010) observed that 14.2% of cervids from southern Spain harbored
70 subcutaneous larvae, whereas the seropositivity determined by indirect ELISA was
71 43.3% and 40.0% for a crude larval extract and a purified antigen, respectively.

72 Due to the increasing incidence of *H. actaeon* in roe deer from Spain since the first
73 citation in 2016, a wide scale retrospective study aims to assess, by means of indirect
74 ELISA, the temporal and spatial spread of this infection in roe deer hunted throughout
75 the Spanish territory.

76

77 MATERIAL AND METHODS

78 Study area and animals

79 This study takes part of a national survey focused on the determination of the main
80 pathogens affecting roe deer in Spain.

81 During a 6-year period (2013-2018), 244 roe deer hunted in different geoclimatic areas
82 of peninsular Spain (Figure 1) were analyzed. Blood samples were taken during field
83 necropsies by intracardiac puncture or from the thoracic cavity by using Venojet® glass
84 tubes without anticoagulant. Sera were obtained by centrifugation and stored at -20°C.

85 Table 1 represents the distribution of the animals considering the different variables
86 included in the study (sampling year, geoclimatic area, sex and age). Age was assessed
87 according to their teeth features (Høy, 2006).

88 ELISA protocol

89 The presence of anti-*Hypoderma* antibodies (IgG) was assessed by an indirect enzyme-
90 linked immunoassay (ELISA) using a crude larval extract (CLE) obtained from L1 of
91 *H. lineatum* collected in the oesophagus of domestic cattle. The antigen preparation and
92 ELISA protocol were performed according to Panadero *et al.* (2010). Briefly, 96-well
93 microplates were coated with 5.5 µg.ml⁻¹ of CLE in phosphate-buffered saline (PBS)
94 pH 7.4 and incubated for 30 min at 37°C. Serum samples were diluted 1:10 in PBS-
95 Tween containing 0.2% skimmed milk and added to duplicate wells. After an
96 incubation period of 30 min. at 37°C a peroxidase-labelled rabbit anti-deer IgG (H+L
97 Accurate Chemical & Scientific Co, Westbury, USA) at 1 µg.ml⁻¹ was used as
98 conjugate. Substrate *O*-phenylenediamine (SigmaFast OPD tablets; Sigma-Aldrich

99 Química, Madrid, Spain) was added to each well and incubated for 20 minutes in
100 darkness at room temperature. The reaction was stopped with 100 μ l per well of 3N
101 H₂SO₂ and absorbances were measured using an ELISA-plate reader (680XR; Bio-Rad
102 Laboratories, California, USA) at 450 nm.

103 Positive and negative control sera were introduced on each plate to normalize
104 absorbance values. The cut-off value for the assay was determined as the mean optical
105 density (OD) of a population of negative sera plus two times the standard deviation (+2
106 SD).

107 **Statistical analysis**

108 Data were processed using Microsoft Excel 2010. Statistical analyses were performed
109 with R statistical language version 3.5.0 (R Core Team, 2018). Variables (year,
110 geoclimatic area, sex and age) were grouped and categorized for statistical analysis as
111 represented in Table 1. Seropositivity was analyzed with a Logistic Regression
112 algorithm. Factors were eliminated from the initial model using a backward conditional
113 method based in AIC value (Akaike Information Criterion) until the best model was
114 built. Odds ratios (OR) with 95% confidence intervals (95% CI) were computed by
115 raising e to the power of the logistic coefficient over the first category of each factor
116 (reference category), not over the last.

117 **RESULTS**

118 The overall seroprevalence in the examined roe deer was 13.9% (34/244).
119 Seroprevalence values considering the different variables included in the study are
120 presented in Table 1.

121 During the 6-year period of study, seroprevalence increased from 10.7% in 2013 to
122 25.9% in 2018. According to the geoclimatic regions, the highest seroprevalences were
123 found in roe deer from continental and mountainous areas of central Spain, followed by
124 those from south-mediterranean area and finally, northern-oceanic region. It is
125 noteworthy that in 2013 all seropositive animals originated from central-continental and
126 south-mediterranean areas, whereas in 2014 seropositive animals were detected in all
127 geoclimatic areas.

128 The percentage of seropositivity against *Hypoderma* was very similar in young and
129 adult animals and slightly higher in females than in males.

130 Logistic regression indicated that roe deer seropositivity against *Hypoderma* was only
131 associated with the geoclimatic area where the animals were hunted ($P<0.05$).

132 Differences were only significant between the central-continental and north-oceanic
133 areas; so that, the risk of being seropositive was 10.5 times higher in roe deer from
134 central-continental area than in those from the north-oceanic one (Table 2).

135

136 **DISCUSSION**

137 The high seroprevalence of antibodies against *Hypoderma* spp observed in roe deer
138 from central Spain, only two years after the first report of larval infestation by *H.*
139 *actaeon* in this host, would indicate a rapid expansion and adaptation of this parasite to
140 roe deer. However, one of the shortcomings of indirect ELISA is the impossibility to
141 identify the species of *Hypoderma* infesting the animals. Considering that all the larvae
142 collected in the last years in roe deer in Spain have been identified as *H. actaeon*
143 (Panadero *et al.*, 2018), and that *H. diana* has not been reported in the last two decades
144 in this country, it can be assumed that the production of anti-*Hypoderma* antibodies in
145 seropositive animals has been induced by the infestation with *H. actaeon*. Moreover,
146 crossreactions due to hypothetical contacts of roe deer with cattle species such as *H.*
147 *lineatum*, should be discarded considering the reduced levels of seropositivity detected
148 in roe deer from the North-oceanic area where grazing cattle is very common and the
149 prevalence of cattle hypodermosis is high (Panadero *et al.*, 2007).

150 A switch to a new host is a very rare event in specific parasites (Pavlásek & Minář,
151 2014). **With the exception of *H. diana* and, the recently rediscovered species, *H. sinense*
152 that infects yaks and cattle in China (Otranto *et al.*, 2004; 2005), larvae of warble fly
153 species are only rarely found on non-specific hosts.** According to Price (1980),
154 specificity in the Oestridae family depends on host availability rather than from strong
155 selection pressures for specialization on one or a few hosts. **Although the first report of
156 infestation by larvae of *H.actaeon* in roe deer was published in 2016 (Panadero *et al.*,
157 2016), the presence of *Hypoderma* spp. specific antibodies in roe deer since at least
158 2013, would indicate the existence of host-parasite contacts prior to this date.** It is
159 noteworthy that in 2013 all seropositive animals were concentrated in two foci located
160 in the Center (Soria province) and the South (Cádiz province) of the Iberian Peninsula,
161 whereas in the following years seropositive animals were detected in all geoclimatic
162 areas. The presence of seropositive animals in two distant areas of the Peninsula may
163 indicate that the host-switch could have occurred independently in both areas.

164 Red deer populations in some areas of central Spain have increased in the last decade
165 due to their introduction in private preserves for hunting purposes. Therefore, the

166 increase of red deer densities in an area where roe deer was the predominant cervid may
167 have favoured sympatry between both species, thus leading appearance of cross
168 infections (Panadero *et al.*, 2016). This hypothesis is supported by Pavlásek & Minář
169 (2014) who affirmed that specific parasites do not switch to a new host when the
170 original one is becoming extinct but only at the time when both the original and the new
171 host are abundant at the same time.

172 A similar situation occurred after the introduction of mouflons in Eastern Europe, where
173 cases of severe mouflon infestations by *H. diana* were reported in areas where both
174 hosts (roe deer and mouflons) were abundant at the same time (Minář, 1982).

175 Roe deer has a homogeneous distribution in northern and central areas of the Iberian
176 Peninsula; however, its distribution is very fragmented in the South and limited by
177 prolonged periods of summer drought, typical of Mediterranean areas, leading to local
178 isolated populations (Braza *et al.*, 1989; Aragón *et al.* 1995). Cádiz forests represent the
179 south-western limit of the distribution of the European roe deer; in this area roe deer and
180 red deer are sympatric (San José *et al.*, 1997). Efficient strategies of conservation and
181 management of these local roe deer populations have achieved to increase roe deer
182 densities that coexist with important populations of red deer.

183 Our results suggest that changes in the pattern of distribution of both red deer and roe
184 deer in different areas of Spain could have favoured the spreading of this myiasis from
185 red deer towards roe deer. Those findings also corroborate the assumption of Pavlásek
186 & Minář (2014) that a switch to a new host or, an attempt at such a switch, by a specific
187 parasite will take place only in a situation when both species are simultaneously present
188 in large numbers.

189 Despite the increment of the seroprevalence, the irregular distribution of the number and
190 origin of samples has led to the absence of statistical differences between years.

191 As previously detected in red deer (Pérez *et al.*, 1995; San Miguel *et al.*, 2001; De la
192 Fuente *et al.*, 2001), no differences in seroprevalence between sexes were found in roe
193 deer. However, contrary to these authors, who observed that the prevalence by *H.*
194 *actaeon* in red deer increase with the age of the host, in our study no differences were
195 found according to the age of roe deer.

196 Although the seroprevalence detected in this ungulate is still lower than that observed in
197 red deer from neighbouring areas (De la Fuente *et al.*, 2001), the mean intensities of
198 parasitisation are considerable (1-60 larvae), **suggesting that roe deer should be**
199 **considered as a suitable host *H. actaeon*** (Panadero *et al.*, 2018). It has been reported

200 that low to medium levels of infection by *H. diana* (max 50 larvae per animal) do not
201 affect the weight of roe deer (Salaba *et al.*, 2013) but severe infestations by this species
202 can be fatal to animals (Yeruham *et al.*, 1994).

203 Our results should alert wildlife authorities on the implications that changes in the
204 population patterns of cervidae can have in the distribution of WF infestations.

205 Moreover, climate changes have a significant effect, not only in the development of the
206 free-living stages of WF (Tarry, 1980) but also in the distribution and abundance of
207 wild cervids. Thus, decreasing severity of winter conditions has led to a population
208 increase and expansion of white-tailed deer (*Odocoileus virginianus*) into the North
209 American boreal forest (Dawe & Boutin, 2016), which are also accompanied by
210 increased diseases spread (Anderson *et al.*, 2015).

211

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319

320 Table 1. Seroprevalence of *Hypoderma* antibodies in roe deer from Spain according to
 321 the different studied factors

| Variable | N° | Positive (%) | 95% CI |
|--------------------------------------|-----|--------------|-------------|
| Year | | | |
| 2013 | 28 | 3 (10.7) | 1.60-30.62 |
| 2014 | 77 | 10 (13.0) | 5.08-25.46 |
| 2015 | 14 | (0.0) | 0-26.76 |
| 2016 | 13 | 1 (7.7) | 0.40-37.91 |
| 2017 | 85 | 13 (15.3) | 8.71-25.10 |
| 2018 | 27 | 7 (25.9) | 11.87-46.59 |
| Geoclimatic area ^a | | | |
| Central-Continental | 54 | 15 (27.7) | 12.79-38.37 |
| North-Oceanic | 57 | 2 (3.5) | 0.61-13.16 |
| Mediterranean | 80 | 9 (11.2) | 5.59-20.76 |
| Mountainous | 50 | 6 (12.0) | 8.29-44.21 |
| Age ^b | | | |
| ≤2 years | 38 | 5 (13.2) | 4.95-28.88 |
| >2 years | 172 | 19 (11.1) | 8.27-19.95 |
| Sex ^c | | | |
| Female | 58 | 9 (15.5) | 7.77-27.92 |
| Male | 161 | 16 (12.6) | 7.60-19.94 |

^a 3 animals without origin; ^b 35 animals without age; ^c 25 animals without sex

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326 Table 2. Results of the logistic regression model of risk factors associated with
 327 seropositivity of *Hypoderma* spp in roe deer from Spain

| | Estimate | Z value | P | OR | CI 95% |
|---------------------|----------|---------|-------|-------|--------------|
| North-oceanic | - | - | - | - | - |
| Mountainous | 1.3218 | 1.571 | 0.116 | 3.75 | 0.818-26.463 |
| South-mediterranean | 1.2487 | 1.557 | 0.119 | 3.48 | 0.855-23.476 |
| Central-continental | 2.3587 | 3.019 | 0.003 | 10.58 | 2.776-69.579 |

328

329

330 Caption of Figures

331 Figure 1. Image of the Iberian Peninsula showing the spatial distribution of sampling
332 sites. Symbol size is proportional to the number of roe deer examined in each location.
333 The percentage of positive samples in each site is represented inside the symbol (in
334 black).

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336