

1 **Parasite-mediated manipulation? *Toxoplasma gondii* infection increases risk**
2 **behaviour towards culling in red deer**

3 Matteo Nava^{1,+}, Luca Corlatti^{2,3,+,*}, Nicoletta Formenti⁴, Tiziana Trogu⁴, Luca Pedrotti², Alessandro
4 Gugiatti², Paolo Lanfranchi¹, Camilla Luzzago^{1,5}, Nicola Ferrari^{1,5}

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6 ¹ Department of Veterinary Medicine and Animal Sciences, University of Milan, Lodi, Italy

7 ² Stelvio National Park – ERSAF Lombardia, Bormio, Italy

8 ³ Chair of Wildlife Ecology and Management, University of Freiburg, Freiburg, Germany

9 ⁴ Experimental Zooprophyllactic Institute of Lombardy and Emilia-Romagna “Bruno Ubertini”,

10 Brescia, Italy

11 ⁵ WildlifeHealth Lab, University of Milan, Lodi, Italy

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17 **+ MN and LC contributed equally to this work**

18 *** Correspondence author:** Luca Corlatti, luca.corlatti@wildlife.uni-freiburg.de;

19 luca.corlatti@ersaf.lombardia.it

20 **Abstract**

21 Parasites can modify host behaviour to increase their chances of survival and transmission.

22 *Toxoplasma gondii* is a globally distributed protozoan whose ability to modify host behaviour is
23 well known in taxa such as rats and humans. Less well known are the effects on the behaviour of
24 wild species, with the exception of a few studies on primates and carnivores. Taking advantage of a
25 culling activity conducted in the Stelvio National Park (Italy), the serological status of *T. gondii* was
26 studied in 260 individuals of red deer *Cervus elaphus* with respect to the risk of being culled. A
27 temporal culling rank index was fitted as response variable, and *T. gondii* serological status as main
28 explanatory variable in linear models, taking into account covariates such as sex, age, jaw length,
29 bone marrow fat and culling location. The overall seroprevalence of *T. gondii* was 31.5%, and the
30 selected models suggested that seropositive deer were culled earlier than seronegative ones, but this
31 effect was only evident in females, in individuals with medium-good body conditions, and in areas
32 with greater human presence. Our results suggest that *T. gondii* may be involved in risk behaviour
33 in large herbivores, supporting its role as a facilitator of predation risk.

34

35 **Key words:** behavioural changes, culling, host manipulation, pathogens, wild ungulates, parasites

36 **Introduction**

37 Several pathogens have developed strategies to increase their chances of transmission within
38 animal populations [1]. One such strategy is the manipulation of host behaviour through direct or
39 indirect mechanisms [2]. The rabies virus, for example, acts directly on the host's nervous system,
40 causing an increase in aggressive behaviour and thus a greater likelihood of biting, thereby
41 facilitating its own transmission [3]. Parasites can also directly influence secondary aspects of the
42 host, such as its metabolism or immune system, thus indirectly its behaviour [2].

43 A parasite known for its direct and indirect impact on host behaviour is *Toxoplasma gondii*.
44 This pathogen acts at the level of the central nervous system, lowering risk perception by decreasing
45 anxiety, fear and vigilance in several species. Attenuated fear response, for example, was observed
46 in rats [4], possibly favouring the transmission of the parasite through predation, while in humans it
47 may cause psychological disorders [5,6]. Most of the studies on the effects and physiological
48 mechanisms of *T. gondii* involving risk-taking behaviour have been obtained in laboratory
49 conditions [7], whereas the alteration of behavioural mechanisms in the wild is understudied.
50 Following infection with *T. gondii*, an increased likelihood of predation by sharks has been
51 observed in sea otters *Enhydra lutris nereis* [8], and increased risk behaviour has been observed in
52 central chimpanzees *Pan troglodytes troglodytes*, spotted hyenas *Crocuta crocuta* and grey wolves
53 *Canis lupus* [9-11]. Overall, however, we have limited knowledge of the impact of *T. gondii* on
54 behaviour in wildlife populations.

55 Taking advantage of a population of red deer *Cervus elaphus* subject to a culling programme
56 [12], assuming individuals seropositive toward *T. gondii* antibodies are infected by tissue cysts [13],
57 we investigate the potential effects of *T. gondii* exposure on altering deer behaviour. In our system,
58 the role of predation is represented solely by culling, as natural predators such as grey wolf were
59 absent at the time of study. We tested the hypothesis that the risk behaviour of seropositive deer is
60 altered, and we expect seropositive individuals to be culled earlier than seronegative ones.

61 **Material and Methods**

62 *Study area and population*

63 The Stelvio National Park is located in the central Italian Alps, between 800 m a.s.l. and 3851 m
64 a.s.l.; at the time of the study, the red deer population in the study area had a density of about 30
65 deer/km² in winter, with an estimated number of deer of about 1400 individuals [14]. Given the
66 impact on forest regeneration and biodiversity, in recent years the Park has decided to reduce deer
67 density through culling in the wintering area, over about 2679 ha. Within the area, 3 culling sub-
68 areas with different levels of anthropisation were identified: low (772 ha), intermediate (1200 ha)
69 and high (707 ha) [11].

70

71 *Data collection*

72 Data were collected during the culling seasons 2016-17 and 2017-18, from the end of November to
73 the end of January. All culled deer ($n = 260$) were brought to a checkpoint. The culling plan
74 included 9% yearling (1.5 years) males, 12% adult (≥ 2.5 years) males, 7% yearling (1.5 years)
75 females, 39% adult (≥ 2.5 years) females and 33% calves (0.5 years).

76 First, shooting date was recorded to generate a ‘culling rank index’ based on the temporal
77 order of culling, from 1 to n (1 if the animal was shot on the first day of each culling season, n if it
78 was shot on the last day of culling: deer culled on the same day were assigned the same rank), for
79 each year separately. The culling rank index is intended as a proxy to investigate the probability of
80 individuals being culled at a given time. Similarly, a rank index to assess possible effects of *T.*
81 *gondii* on the host has been used in wild brown rats *Rattus norvegicus* [15], where the probability of
82 being trapped was found to be positively associated with *T. gondii* seropositive status., i.e., the rats
83 that tested serologically for *Toxoplasma* were those that were caught earlier.

84 Next, assuming that individuals testing positive to IgG antibody toward *T. gondii* (but see
85 [13]), are infected, we investigated its occurrence in deer through ELISA [Enzyme-Linked
86 ImmunoSorbent Assay] test (ID Screen® Toxoplasmosis Indirect Multi-species ELISA, IDVET,

87 Montpellier, France) after collecting blood from the jugular vein. During the inspection at the
88 checkpoint, the veterinary staff assessed the health conditions of animals in order to exclude co-
89 morbidities that might influence the behaviour of deer, and thus their probability of being culled. To
90 control for other potential confounding effects in the relationship between culling rank index and *T.*
91 *gondii* seropositivity, for each animal we collected data on sex, age, jaw length, eviscerated body
92 mass, proportion of bone marrow fat (measured through the dehydration of the marrow of
93 metacarpals [16]) and sub-area of culling. Jaw length is a good indicator of skeletal development
94 [17], while eviscerated body mass and proportion of bone marrow fat are representative indices for
95 the nutritional and body conditions of the animal. As body mass and body condition both decline
96 over winter due to food restrictions, to avoid bias, for each year these variables were adjusted to the
97 first day of culling by fitting quadratic linear models between body mass / bone marrow and Julian
98 date, from the first day of culling, for different age-classes, for the two sexes separately.

99

100 *Statistical analyses*

101 To investigate the relationship between temporal rank of culling and *T. gondii* seropositivity status,
102 a regression modelling approach was used, where the global model (*sensu* [18]) included all the
103 collected variables (and their first order interactions) that were considered to be related, and explain,
104 *T. gondii* seropositive status. Culling rank index was the response variable, and *T. gondii* serological
105 status (negative/positive) was the main explanatory variable in interaction with sex, age-class, jaw
106 length, proportion of bone marrow fat, level of anthropisation of culling area and year of culling.
107 Such first-order interactions are considered important in order to reveal subtle effects of *T. gondii*
108 which might affect behaviour only in specific condition. Body mass was excluded because of
109 collinearity issues detected during preliminary data analyses. As seropositive red deer to *T. gondii*
110 does not generate obvious external signs of disease in red deer, culling was assumed to be unbiased.

111 Since the response variable was a rank variable, ordered regression would be the natural
112 approach to modelling. However, an alternative approach is to use simple linear regression on

113 transformed ranks, as long as the number of categories is large [19], as in our case. After
114 preliminary analyses, a boxcox function showed improved model fit after a square-root
115 transformation for the response. Our full model was thus of the form:

$$116 \quad CRI_i \sim N(\mu_i, \sigma^2)$$

$$117 \quad E(CRI_i) = \mu_i \quad \text{and} \quad \text{var}(CRI_i) = \sigma^2$$

$$118 \quad \mu_i = \text{infection status}_i \times (\text{sex}_i + \text{age}_i^2 + \text{bone marrow fat}_i^2 + \text{jaw length}_i^2 + \text{anthropisation level}_i + \text{year}_i)$$

119 where CRI_i was the square-root value of the culling rank index for observation i , assumed to be
120 normally distributed with expected value μ_i and variance σ^2 . The adequacy of the full model was
121 inspected through residual diagnostics. To obtain a more conservative model structure, model
122 selection was conducted through best subsets regression, based on the minimisation of the AICc
123 values. Unnested models within delta AICc ≤ 2 were averaged to obtain final parameter estimates.
124 In presence of interactions, post-hoc pairwise comparisons were conducted using Bonferroni
125 adjustment for multiple contrasts. To further assess the consistency of our results, a similar analysis
126 using an ordinal regression modelling approach on untransformed data, as well as a path analysis
127 were also performed (Supplementary 1). All analyses were conducted with R [20] in RStudio [21].

128

129 **Results**

130 A total of 260 individuals (162 females and 98 males) were culled, 82 of which (59 females and 23
131 males) tested positive to *T. gondii* antibodies, leading to a mean prevalence of 31.5% (95% CI:
132 26.1-37.3).

133 The global model did not show major violations of assumptions. After model selection, 4
134 unnested models were considered plausible to explain variation in culling rank index. These
135 included the interactions of *T. gondii* serological status with sex, level of anthropisation and
136 proportion of bone marrow fat (plus their lower terms), as well as the effect of culling year (table 1).
137 The results suggested that seropositive deer, in general, were not culled earlier than seronegative
138 ones (the main effect of *T. gondii* was non-significant), but the effect was evident in females (table 2

139 and figure 1a), in individuals with medium-good body conditions (table 2 and figure 1b), and in
140 areas with greater human presence (table 2 and figure 1c). Furthermore, in 2017-18 deer were
141 culled earlier than in 2016-17 (table 2 and figure 1d). Specifically, with respect to the interaction
142 between *T. gondii* serological status and sex, culling rank index was significantly different between
143 *T. gondii*-negative (n = 103) and *T. gondii*-positive (n = 59) female deer (difference = 0.805, *p*-value
144 = 0.003), but not between *T. gondii*-negative (n = 75) and *T. gondii*-positive (n = 23) males
145 (difference = 0.114, *p*-value = 0.695). Culling rank index was significantly different between
146 negative females and negative males (difference = 0.505, *p*-value=0.003), but not between positive
147 females and positive males (difference = -0.187, *p*-value = 0.527) (figure 1a). With respect to the
148 interaction between *T. gondii* serological status and level of anthropisation, culling rank index was
149 significantly different between *T. gondii*-negative (n = 74) and *T. gondii*-positive (n = 44) deer in
150 the highly anthropised area (difference=0.813, *p*-value=0.002) but not between *T. gondii*-negative
151 (n = 53) and *T. gondii*-positive (n = 25) deer in the areas with low level of anthropisation (difference
152 = -0.003, *p*-value = 0.990), nor between *T. gondii*-negative (n = 51) and *T. gondii*-positive (n = 13)
153 deer in the areas with medium level of anthropisation (difference=0.570, *p*-value=0.133). No
154 difference occurred across different levels of anthropisation both in negative (low-medium = 0.111,
155 *p*-value = 0.862; low-high = -0.338, *p*-value = 0.203; medium-high = -0.450, *p*-value = 0.069) and
156 in positive deer (low-medium = 0.684, *p*-value = 0.161; low-high = 0.478, *p*-value = 0.196;
157 medium-high = -0.206, *p*-value = 0.824) (figure 1c).

158 The same results were obtained using an ordinal regression modelling approach on
159 untransformed ranks (Supplementary 1), supporting the fact that a linear model to the square root of
160 ranks did not create any artefacts. Furthermore, a path analysis on square-root transformed ranks
161 confirmed the occurrence of direct effects only, thereby supporting the linear model results
162 (Supplementary 1).

163

164 **Discussion**

165 In our study, culling rank index was not affected by the occurrence of *T. gondii* serological
166 status alone, as seropositive deer were not culled earlier than negative ones. A statistically
167 significant effect was nonetheless evident when *T. gondii* serological status was in interaction with
168 other factors such as sex, body condition and level of anthropisation. Furthermore, deer were culled
169 earlier in 2017-18 than in 2016-17. Previous studies conducted in laboratory settings have shown
170 that *T. gondii* infection can manipulate the intermediate host behaviour through changes in
171 neurotransmitters [7]. This, in turn might increase boldness and possibly facilitate predation by the
172 definitive host [17, 22-25]. Using a similar approach than the one used in this study, [14] showed
173 that the action of *T. gondii* led to infected rats being trapped earlier than non-infected ones, thereby
174 supporting a potential ‘parasite increased susceptibility to predation’. This form of parasite
175 manipulation increases predation on intermediate hosts [26] also in wildlife species, as recently
176 observed in hyenas and primates [9,10].

177 Wildlife populations are characterized by great behavioural variability among individuals,
178 including intersexual differences owing to differences in the trade-offs between energetic needs and
179 risk avoidance [27,28]. For example, it may be argued that female deer are naturally shyer than
180 males, i.e., they tend to show more cautious behaviour and occupy forested areas more than males,
181 possibly reflecting an antipredator strategy associated to their reproductive role [29]. As observed in
182 rats, where *T. gondii* infection is thought to induce behavioural change through modulation of
183 neurotransmitters and hormones involved in stress regulation [7], in red deer *T. gondii* infection
184 might have favoured a relaxation of the perception of risk in females, thereby leading to the
185 adoption of a bolder behavior. Such a behaviour may manifest itself through increased use of open
186 areas, with consequent higher chance of being culled. This effect was not observed in males, but we
187 cannot rule out the impact of limited sample size. According to figure 1b, *T. gondii* seropositive
188 individuals with medium-good body conditions appeared to be culled earlier than individuals with
189 poorer body conditions. Given the limited data for individuals with lower proportion of bone
190 marrow (n = 7 with bone marrow values < 0.5), any interpretation requires caution: with this caveat

191 in mind, it might be that the mild effects of *T. gondii* are less evident in animals facing poor
192 conditions because these effects are overwhelmed by stronger factors acting on animal such as
193 asthenia and a lower propensity for movement (induced by starvation). On the other hand, an
194 artificial selection operated by hunters, willing to obtain individuals in better body conditions may
195 also explain the pattern, but as *T. gondii* does not affect body conditions it is unlikely that hunters
196 may select for serological status deer.

197 *T. gondii* seropositive deer were culled earlier than negative ones in areas with high presence
198 of humans. This pattern may be due to the fact that uninfected deer in highly anthropised areas tend
199 to be shy for antipredator reasons [30], thus are culled later than the seropositive ones, as the latter
200 might have become risk-prone following exposure with *T. gondii*. This suggestion is supported by
201 the absence of difference between serological status of deer in medium- and low-anthropised areas,
202 where deer may normally be less cautious due to low human presence. Furthermore, in anthropized
203 areas, the greater presence of domestic cats *Felis silvestris catus*, the final host of *T. gondii*, might
204 increase the prevalence [31] and, possibly, the population effects on deer behaviour. With respect to
205 difference between years, a weather station in the proximity of the study area, at 2300 m a.s.l.,
206 showed that the mean daily snow cover was 86.3 cm in December 2017 and 54.8 cm in December
207 2016 [32]. This may explain the observed year-effect on culling rank index, as a greater snow cover
208 constrains deer movement to lower elevations, increasing deer density and favouring culling
209 probability.

210 We are aware that a number of potential unmeasured variables could explain the relationship
211 between *T. gondii* serological status and culling rank index. Our models accounted for several
212 internal and external variables. Furthermore, during the veterinary inspection at the checkpoint, no
213 animal showed pathological signs with possible effect on behaviour, e.g., movement disability or
214 impaired organ functionality, therefore the culling rank is unlikely to be biased by clinical co-
215 morbidities. Other infections were detected in our study population. For example, red deer are
216 asymptomatic reservoir of Shiga toxin-producing *Escherichia coli* and *Staphylococcus aureus*,

217 which may have zoonotic potential [33,34], but none of them are related to the probability of being
218 culled.

219 Our study relies on the assumption that all infected animals are seropositive. However, it
220 should be pointed out that, in the case of antibody waning, it would be in fact the animals that are
221 infected for the longest that may be tested as seronegative. Taking this caveat into account, this
222 study shows some evidence for possible behavioural effects of *T. gondii* in large herbivores under
223 natural conditions. To our knowledge, the only other study investigating potential behavioural
224 effects of *T. gondii* in herbivores was recently conducted on domestic sheep *Ovis aries*, providing
225 evidence of a relationship between *T. gondii* infection and problem-solving behaviour, but no
226 alteration of fear response [35]. In our study area, there are no large felines that can prey on deer,
227 and the only species in which the parasite can complete its cycle is the domestic cat. More
228 generally, however, modification of prey behaviour toward higher risk of predation could have
229 evolutionary evolved to enhance the transmission to definitive hosts such as lynx *Lynx lynx* in
230 Europe, or bobcat *Lynx rufus* and cougar *Puma concolor* in North America. In this respect, given
231 the current expansion of the wolf on the Alpine territory, it may be expected that *T. gondii* exposure
232 could become a possible facilitator of predation of deer by wolf. This ideally requires investigation
233 of the predation risk in relation to *T. gondii* positive deer, and possibly of the associated cascading
234 effects on the other ecosystem components.

235

236 **Data accessibility**

237 Dataset and R-Script used for this analysis are available as supplementary material. Metadata are
238 included in the R-Script.

239

240 **Acknowledgements**

241 We would like to thank A. Zanolli for his invaluable help in collecting data.

242 **Table 1** Model selection for the effects of human presence (Hp), body condition (Bc), sex (Sex),
 243 toxoplasma serological status (Ts), year (Year) and their interactions on culling rank index. The table
 244 reports degrees of freedom (df) differences in Akaike's Information Criterion corrected for small
 245 sample size (ΔAIC_c) between each model and the model with the lowest AIC_c and the Akaike's
 246 weights (Weight). The '+' indicates variables included in the models. Only models with delta ΔAIC_c
 247 value ≤ 2 are reported.

Hp	Bc	Sex	Ts	Year	Hp × Ts	Bc × Ts	Sex × Ts	df	AIC_c	ΔAIC_c	Weight
+	+	+	+	+	+	+	+	14	797.8	0.00	0.428
	+	+	+	+		+	+	10	799.4	1.53	0.199
+	+	+	+	+		+	+	12	799.4	1.61	0.191
+	+	+	+	+	+			11	799.6	1.72	0.181

248

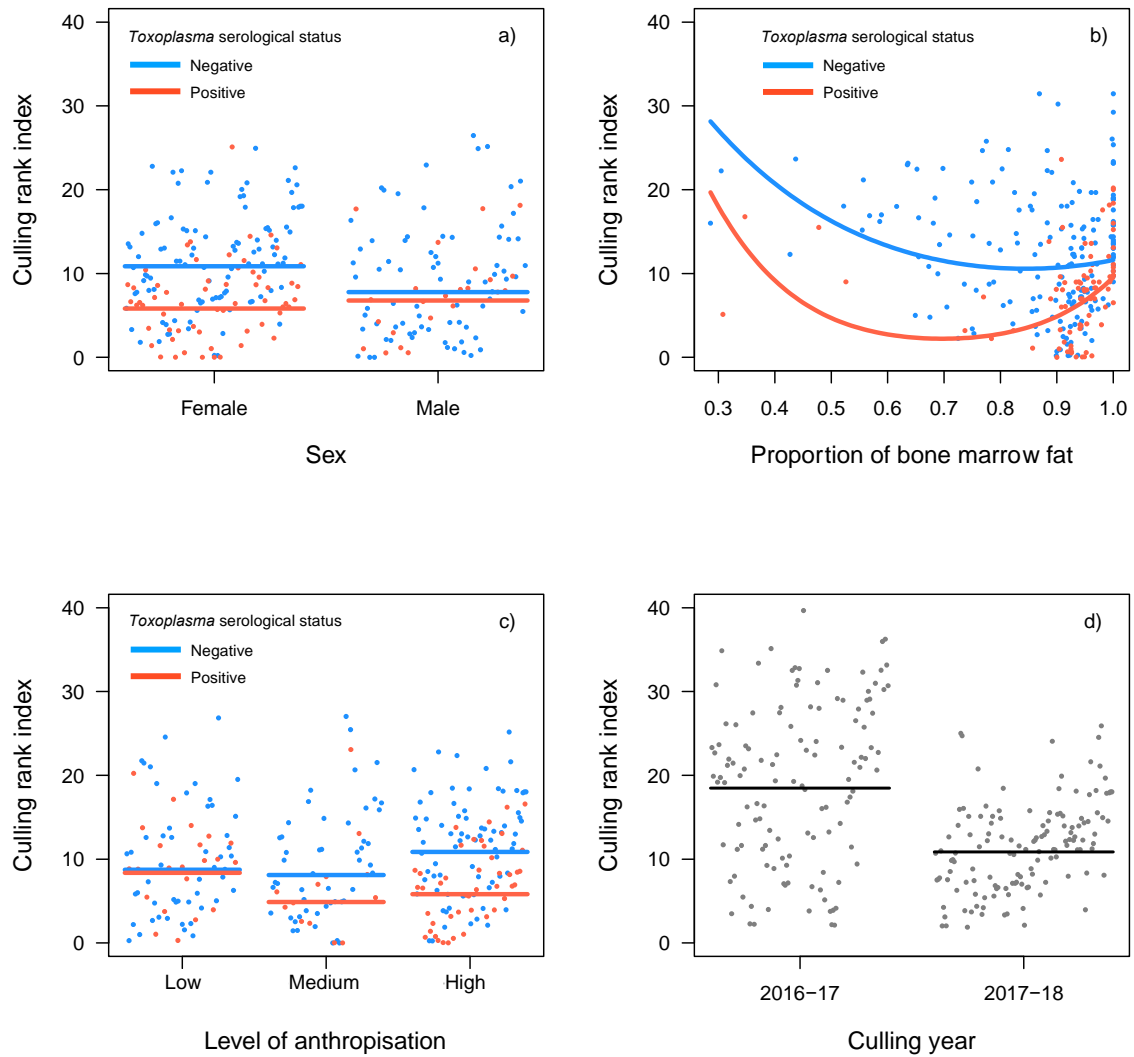
249 **Table 2** Averaged parameter estimates of the models selected to explain the variation in culling rank
 250 index of red deer in the winter of 2016-17 and 2017-18 in the Stelvio National Park. The table reports
 251 parameter estimates with associated lower and upper bounds of 95% confidence interval (95LCL and
 252 95UCL, respectively).

253

Parameter	Coefficient	SE	95LCL	95UCL
(Intercept)	4.086	0.184	3.724	4.447
<i>T. gondii</i> serological status [Positive]	-0.266	0.403	-1.056	0.525
Sex [Male]	-0.477	0.175	-0.819	-0.135
Proportion of bone marrow fat	-2.896	1.437	-5.712	-0.079
Proportion of bone marrow fat ²	3.181	1.321	0.592	5.770
Level of anthropisation [Medium]	-0.154	0.222	-0.589	0.281
Level of anthropisation [High]	0.275	0.225	-0.166	0.716
Year [2017-18]	-0.995	0.141	-1.270	-0.719
<i>T. gondii</i> serological status [Positive] × Sex [Male]	0.673	0.343	0.000	1.346
<i>T. gondii</i> serological status [Positive] × Proportion of bone marrow fat	6.718	2.837	1.158	12.278
<i>T. gondii</i> serological status [Positive] × Proportion of bone marrow fat ²	4.697	2.765	-0.723	10.117
<i>T. gondii</i> serological status [Positive] × Level of anthropisation [Medium]	-0.553	0.434	-1.404	0.298
<i>T. gondii</i> serological status [Positive] × Level of anthropisation [High]	-0.817	0.341	-1.486	-0.148

254

255 **Figure 1** Marginal effects of the variables selected to explain the variation in culling rank index of
256 red deer in the winter of 2016-17 and 2017-18 in the Stelvio National Park: a) sex; b) proportion of
257 bone marrow fat; c) level of anthropisation by serological status; d) year of culling.



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