Mycoplasma conjunctivae in insect vectors and anatomic locations related to transmission and persistence

Xavier Fernández Aguilar[a,b*], Jorge Ramón López-Olvera[a], Maria Puig Ribas[a], Mattia Begovoeva[a], Roser Velarde[a], Jesús Cardells[a,c], Oscar Cabezón[b,a].

[a] Wildlife Ecology & Health group - Servei d' Ecopatologia de Fauna Salvatge (SEFaS), Departament de Medicina i Cirurgia, Universitat Autònoma de Barcelona, Bellaterra 08193, Spain.

[b] UAB, Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, Bellaterra 08193, Spain.

[c] Universidad Cardenal Herrera-CEU, CEU Universities, Avda. seminario s/n, Moncada 46113 Valencia, Spain.

***Corresponding author**: Xavier Fernández Aguilar. email: xfdezaguilar@gmail.com

Declarations of interest: None.

22 **Abstract**

23 *Mycoplasma conjunctivae* is an obligate microparasite that causes Infectious Keratoconjunctivitis
24 (IKC) in Caprinae species. IKC is a long-recognised disease, but little attention has been paid to the
25 mechanisms of transmission of the mycoplasma and its occurrence in locations other than the eyes.
26 In this study, the presence of *M. conjunctivae* is assessed in the eyes, external ear canals (EEC),
27 nasal cavity, and vagina of host species as well as in potential vectors, which may be involved in the
28 transmission and persistence of infection within the host.

29 *M. conjunctivae* was detected by qPCR in 7.2 % (CI 95% 4.7-11.0) of the ear swabs and 9.5 % (CI
30 95% 6.4-13.9) of the nasal swabs from Pyrenean chamois, Iberian ibex, domestic sheep and
31 mouflon without statistical differences between species. Mycoplasma detection in nasal swabs was
32 mostly associated with ocular infection (95.6%), but this was not the case for EEC (52.6%). Among
33 the eye-positive ruminants, 27.3% were positive in ear swabs and 64.7% in nasal swabs, and the
34 threshold cycle values of the qPCR were correlated only between eye and nasal swabs ($p<0.01$;
35 $r_2=0.56$). *M. conjunctivae* was detected in 1.7% - 7.1 % of *Musca* spp. captured during an IKC
36 outbreak in Iberian ibex and in one out of three endemic sheep flocks.

37 The results indicate that the transmission of *M. conjunctivae* may occur by direct contact with eye
38 or nasal secretions and/or indirectly through flies. The *M. conjunctivae* DNA detection in EEC
39 suggests that it can colonise the auditory tract, but the significance for its persistence within the host
40 should be further assessed.

41

42 **Keywords:** chamois; disease transmission; external ear canal; flies; Iberian ibex; Infectious
43 Keratoconjunctivitis; vectors; mouflon; Musca; sheep.

1. Introduction

Mycoplasmas are obligate microparasites without cell walls with a short-lived persistence in the environment. Each mycoplasma species of importance to veterinary or human health has a tissue tropism that is generally linked to the main pathology and clinical signs. However, the occurrence of *Mycoplasma* spp. in tissues or organs other than its preferred sites is not rare (Gómez-Martín et al., 2012). Mycoplasmas have been isolated in high numbers and diversity from external ear canals (EEC) in both domestic (Cottew and Yeats, 1982) and wild hosts (González-Candela et al., 2007). *M. ovipneumoniae* and *M. bovis* can occasionally cause otitis media and interna (Besser et al., 2008; Maeda et al., 2003), yet persistence in the EEC may results in carriers without clinical signs (Cottew and Yeats, 1982; DaMassa and Brooks, 1991). In fact, ear carriers of *M. mycoides*, *M. capricolum* and *M. agalactiae* are suggested to be important for persistence and re-emergence of contagious agalaxia in goats (Mercier et al., 2007; Tardy et al., 2007).

Mycoplasma conjunctivae is the primary agent of Infectious keratoconjunctivitis (IKC) in wild and domestic Caprinae species (Fernández-Aguilar et al., 2017; Giacometti et al., 2002). It invades ocular structures and can cause severe clinical signs that can hamper visual performance and eventually lead to the perforation of the cornea (Mayer et al., 1997). It is assumed that *M. conjunctivae* exclusively affects ocular tissues (Mayer et al., 1997). However, its occurrence in other anatomical locations has not been properly assessed with a culture-independent method, which is the most sensitive approach for the detection of mycoplasmas (Amores et al., 2010; Vilei et al., 2007).

The spatio-temporal patterns of IKC epidemics show a rapid spread of the disease (Degiorgis et al., 2000; Gelormini et al., 2017) and eye-frequenting flies have been proposed to play a role in transmission (Degiorgis et al., 1999; Giacometti et al., 2002). Some fly species feed from ocular and nasal secretions of ruminants and are known to be involved in the contagion of bovine IKC,

68 caused by *Moraxella bovis* (Glass and Gerhardt, 1984). However, the vector-borne transmission of
69 *M. conjunctivae* has yet to be assessed.

70 With the aim to investigate transmission mechanisms of *M. conjunctivae* and potential anatomical
71 locations for its persistence within the host, we studied its presence, using a molecular-based
72 technique, in the eyes, EEC, nasal cavity, vagina and in flying insects associated with different IKC
73 epidemiological scenarios. We hypothesise that *M. conjunctivae* occurs in vectors and in locations
74 in the hosts other than the eyes, as yet unreported.

75 **2. Materials and Methods**

76 *2.1. Study areas and sample collection*

77 A convenience sample of 303 wild and domestic ruminants was performed by collecting ocular
78 swabs from beneath the lower eyelid, ear swabs combining both EEC (one swab per animal) and
79 nasal swabs from both nostrils (one swab per animal). A complete set of these samples was
80 obtained from 228 ruminants with further vaginal swabs collected from 70 of the females. All the
81 animals were minimally sampled for eye swabs and swabs from another location (Table 1).

82 Pyrenean chamois (*Rupicapra p. pyrenaica*) and European mouflon (*Ovis aries musimon*) were
83 sampled during the regular hunting season in the National Game Reserves (NGR) of Freser-
84 Setcases and Muela de Cortes, north-eastern and eastern Spain, respectively. Iberian ibex (*Capra*
85 *pyrenaica*) were sampled during routine handling procedures in a captive population that was
86 undergoing a severe IKC outbreak in Sierra Nevada, southern Spain (Fernández-Aguilar et al.,
87 2017b). Five domestic sheep flocks with a known endemic status of asymptomatic *M. conjunctivae*
88 infections were sampled in Bellaterra, la Garriga, Molló, Puiggròs and Térmens municipalities, all
89 in the Catalonia region in north-eastern Spain (Fernández-Aguilar et al., 2013). All swabs were
90 frozen within less than 12 hours after collection and stored at -20ºC until analysis.

91 Flying insects from three of the sheep farms sampled and from the Iberian ibex enclosure were
92 captured with a butterfly net and placed in sterile containers. Insects were captured in close

93 proximity/contact with or in the stalls of the animals. The effort of capture performed was similar at
94 each sampling site but differences in flying insect abundance resulted in a different number of
95 specimens captured (Fig. 1). The insects were directly stunned in the freezer and were stored at -
96 20ºC until identification and analysis.

97 *2.2. Sample preparation and M. conjunctivae detection*

98 The ear, nasal and vaginal swabs were placed in sterile tubes with 500 µl of phosphate-buffered
99 saline (PBS) and subsequently vortexed for one minute. Flies were placed in sterile tubes in pools
100 of three individuals from the same species/genus with 600 µl of PBS or individually with 200 µl of
101 PBS in the case of a low number of individuals (Table 2). Homogenates of flies were then
102 mechanically obtained with a tissue grinder pestle and subsequently vortexed for one minute. The
103 nucleic acids from the ear, nasal and vaginal swabs and the fly homogenates were purified with a
104 commercial kit based on magnetic-particle technology (MagAttract® 96 *cador*® Pathogen Kit,
105 Qiagen Inc.) and the workstation 96 Biosprint (Qiagen Inc.).

106 The eye swabs were placed in sterile tubes with 500 µl of lysis buffer (100 mM Tris–HCl, pH 8.5,
107 0.05 Tween 20, 0.24 mg/mL proteinase K). They were mixed with a vortex for one minute and the
108 cells were lysed for 60 minutes at 60ºC. The inactivation of proteinase K was subsequently
109 performed at 97ºC for 15 minutes.

110 The detection of *M. conjunctivae* DNA was performed on the eye swab lysates and the purified
111 DNA from the ear, nose and fly homogenates with a qPCR protocol previously described (Vilei et
112 al., 2007). Briefly, each reaction consisted of 2.5 µl of the sample, 900 nM of LPPS-TM-L primer
113 and LPPS-TM-R, 300 nM of LPPS-TM-FT probe, 12.5 µl of TaqMan®2x Universal PCR
114 MasterMix (Applied Biosystems, Warrington, UK), an exogenous internal positive control (IPC;
115 Applied Biosystems, Warrington, UK) and water up to 25 µl of volume. Positive and negative
116 controls were included in each plate. The threshold was set at 0.05 and the cycle number when the
117 fluorescence crossed the threshold was recorded as the threshold cycle (Ct) value. The detection

limit was established at a Ct value of 39 to detect low concentrations of the target DNA, consistent with a single mycoplasma cell in the reaction (Ryser-Degiorgis et al., 2009; Vilei et al., 2007).

2.3. Data analyses

Differences among species and anatomic locations were assessed with a two-sided chi-squared test for independence. Differences of Ct values of the *M. conjunctivae*-qPCR between ear and nasal swabs were pairwise compared with a Wilcoxon signed-rank test for non-parametric distributions using the Bonferroni correction. The correlation of Ct values obtained in the eye, nasal and ear swabs was assessed with a non-parametric Spearman correlation test that provides a coefficient (r_s) as a measure of the strength of the relationship. Significance was set at a p-value of 0.05 for all tests. The confidence intervals (CI) of the apparent prevalences were calculated with the "EpiR" package, and the graphics were performed with the "ggplot2" package, all in R statistical software (R Development Core Team 3.4.3, 2017).

3. Results

Mycoplasma conjunctivae DNA was detected in the eye, ear and nasal swabs in all the ruminant species tested, but not in vaginal swabs (Table 1). The overall prevalence of *M. conjunctivae* in the ear swabs was 7.2% (19/264; CI 95% 4.7-11.0), but only 52.6% (10/19; CI 95% 31.7-72.7) of the ear-positive animals were also positive in the eyes. Mycoplasma was detected in the EEC in three flocks out of five sampled, with a within-flock prevalence that ranged from 8.7% (CI 95% 2.4-26.8) to 16.7% (CI 95% 0.8-56.4). The overall prevalence of *M. conjunctivae* DNA in nasal swabs was 9.5% (23/241; CI 95% 6.4-13.9) and were detected in 95.6% (22/23; CI95% 79.0-99.8) of samples when *M. conjunctivae* was also detected in the eyes. Mycoplasma was detected in nasal swabs in three out of four sheep flocks sampled, with a within-flock prevalence that ranged from 3.3% (CI 95% 0.2-16.7) to 30.4% (CI 95% 5.6-50.9).

Among the eye-positive ruminants, 27.3% (9/33; CI 95% 15.1-44.2) and 64.7% (22/34; CI 95% 47.9-78.5) were also positive in ear and nasal swabs, respectively. If considering only animals with

143 Ct values lower than 35 in the eyes, approximately 3500 *M. conjunctivae* cells per sample (Vilei et
144 al., 2007), the concurrent detection of *M. conjunctivae* in ear swabs slightly increased to 33.3%
145 (8/24; CI 95% 17.9-53.3), and in nasal swabs increased to 84.0% (21/25; CI 95% 65.3-93.6).

146 *M. conjunctivae* prevalence in the different anatomical locations was not statistically different
147 among the species sampled if compared separately based on whether or not infection was present in
148 the eyes. However, the median Ct values and its range were significantly ($p<0.01$) higher in the ears
149 swabs (median 38.0; min. 29.7-max. 38.8) as compared to the nasal swabs (33.1; 28.9-37.4) (Fig.
150 2). Ct values of nasal and eye swabs were correlated with a moderate monotonic relationship
151 ($p<0.01$, $r_2 =0.56$), but Ct values of ear and eye swabs were not ($r_2=0.11$).

152 A total of 472 flying insects from the orders Diptera and Coleoptera, and the families Muscidae,
153 Sarcophagidae, Fanniidae and Carabidae, were captured (Table 2). *M. conjunctivae* was detected
154 only in flies from the genera *Musca* associated with the outbreak of IKC in the captive Iberian ibex
155 and with the sheep flock with the highest *M. conjunctivae* eye-prevalence (Table 2). Ct values
156 obtained in positive-qPCR flies were 35.8 and 37.9 in two specimens, consistent with DNA of
157 $2.4x10^3$ and $5.5x10^2$ mycoplasma cells, respectively.

158 **4. Discussion**

159 Infectious Keratoconjunctivitis is a long-recognised disease of wild and domestic ruminants, yet
160 little effort had been devoted to understanding *M. conjunctivae* transmission and its persistence
161 within the host. To the author's best knowledge, this study describes for the first time the presence
162 of *M. conjunctivae* in EEC and flies, providing relative frequencies of mycoplasma DNA
163 occurrence in different body locations.

164 *M. conjunctivae* was more frequently detected in the eyes than any other location, supporting its
165 assumed tropism to ocular structures. Our results also suggest that during ocular infections, *M.*
166 *conjunctivae* commonly colonise EEC in all the species sampled, similarly to that described for
167 other mycoplasmas (Amores et al., 2010; González-Candela et al., 2007). However, the detection of

168 *M. conjunctivae* DNA in EEC was not always associated with its presence in the eyes and indicates
169 the importance of this alternative location for mycoplasma persistence within the host. The
170 longitudinal detection of *M. agalactiae*, *M. mycoides* and *M. capricolum* in EEC during a period of
171 4 to 10 months supports this hypothesis (Cottew and Yeats, 1982). In this sense, the prevalence of
172 pathogenic mycoplasmas and *M. mycoides* subsp. *mycoides* LC (24%-32%) in the ear canals of
173 goats was also reported to be higher in herds with previous disease history than those without.
174 (Mercier et al., 2007; Tardy et al., 2007). It has been proposed that ear canals have lower immune
175 pressure and thus provide a good niche for mycoplasma persistence (DaMassa and Brooks, 1991).
176 Accordingly, the occurrence of *M. conjunctivae* auricular carriers may contribute to its maintenance
177 in host populations, especially in those where it occurs endemically and with a low eye prevalence
178 (Fernández-Aguilar et al., 2017a; Mavrot et al., 2012).

179 The detection of *M. conjunctivae* DNA in the auditory system raises the question as to whether this
180 mycoplasma can also colonise middle and inner ears and, eventually, cause otitis as described for
181 *M. bovis* and *M. ovipneumoniae* (Besser et al., 2008; Maeda et al., 2003). The circling behaviour
182 sporadically observed in severely IKC-affected wild Caprinae is compatible with a vestibular
183 syndrome and worth further investigation (Degiorgis et al., 2000; Giacometti et al., 2002).

184 The consistent detection of *M. conjunctivae* DNA among nasal and eye swabs and the positive
185 correlation of Ct values of these two sites suggests that the nasal cavity is most probably an
186 excretion route through the natural anatomic communication with the eyes, but not a location for
187 the persistence of the mycoplasma. According to the relatively low Ct values detected in nasal
188 swabs, nasal secretions are probably an important source for *M. conjunctivae* transmission. This is
189 in agreement with the early reports of *M. conjunctivae* in nasal smears of sheep, and the high
190 loads—up to 10^7 CFU/ml—isolated from the nasopharynx in experimentally infected sheep
191 (Dagnall, 1993). However, direct comparison of the Ct values between eye and nasal swabs can not
192 be performed because of the different methods used for the molecular detection.

193 This study confirms the presence of *M. conjunctivae* in flying insects found around hosts and
194 strongly suggests that IKC is also a vector-transmitted disease, as generally suspected (Giacometti
195 et al., 2002). Mechanic transmission of ocular pathogens by flies has been demonstrated to occur by
196 regurgitations of the crop of the flies and by superficial contact (Glass and Gerhardt, 1984). Given
197 that direct contact between different host species is rare in alpine pastures (Ryser-Degiorgis et al.,
198 2002), flies may play a major role in cross-species transmission and hence in IKC outbreaks at the
199 wildlife-livestock interface (Belloy et al., 2003; Fernández-Aguilar et al., 2017a). The contribution
200 of vectors for the transmission of *M. conjunctivae* is necessarily dependent on their abundance and
201 dynamics, but our results also suggest that the presence of severe IKC, typically in epizootics, may
202 enhance transmission by flies. Severe IKC is associated with higher *M. conjunctivae* eye-loads and
203 more severe clinical signs and eye discharge (Fernández-Aguilar et al., 2017b; Mavrot et al., 2012),
204 which attract a high number of flies to feed (Fig. 1). This is consistent with the higher prevalence of
205 *M. conjunctivae* DNA detected in flies associated with the Iberian ibex outbreak than in endemic
206 sheep flocks where *M. conjunctivae* infections are mostly asymptomatic (Fernández-Aguilar et al.,
207 2013; Fernández-Aguilar et al., 2017).

208 Insights on the diversity of eye- and carcass-frequenting insects in wild and domestic ruminants
209 indicated that up to four former genera of Muscidae (*Hydrotaea*, *Musca*, *Morellia* and *Polietes*)
210 might be involved in IKC epidemiology in the Swiss Alps (Degiorgis et al., 1999). In the present
211 study, we found lower diversity but also different genera of Diptera, among which *M. conjunctivae*
212 was only detected in *Musca* spp. Some specimens not included in this study but captured at the
213 same time and locations were identified as *Musca domestica* (sheep flocks) and *Musca autmnalis*
214 (Iberian ibex enclosure). The feeding habits of *Musca* spp. on lacrimal and other body secretions
215 (Glass and Gerhardt, 1984) strongly suggests that this genus of flies are relevant vectors for IKC in
216 Caprinae.

217 Further assessment of the viability of *M. conjunctivae* cells should be performed to confirm the
218 relevance of these findings, but the results strongly suggest that *M. conjunctivae* can colonise the

EEC, a location where other mycoplasmas have been shown to persist for long time periods. The epidemiological implications are not clear but auricular carriers may play an important role in mycoplasma persistence. Whether *M. conjunctivae* can cause otitis requires further study. The quantified DNA detection in EEC, nasal cavity and flies unveils potential transmission mechanisms of *M. conjunctivae*, which may occur by direct or close contact through ocular or nasal secretions and indirectly through flies. Vector-transmission of *M. conjunctivae* may enhance contagion in low-density and/or spatially-structured Caprinae populations and is probably a major component for cross-species transmission.

Acknowledgements

We are grateful to Sandra Talavera, Miguel Carles-Tolrá and Andrian Pont for assistance in insect identification. We are also grateful to the directors and rangers of the National Game Reserves and to farmers that kindly agreed to collaborate on this study. We would also like to thank to Joachim Frey for the quantified positive controls provided and the support of several colleagues from the SEFaS research group, veterinary students from UAB and Victor Lizana and Ángel Gómez-Martín from UCH-CEU that occasionally assisted in sample collection. The study was partially funded by the research projects CGL2009-11631 and CGL2012-40043- C02-02 of the Spanish MICINN.

Conflict of interest statement

The authors declare no conflicts of interest.

References

Amores, J., Corrales, J.C., Martín, Á.G., Sánchez, A., Contreras, A., de la Fe, C., 2010. Comparison of culture and PCR to detect *Mycoplasma agalactiae* and *Mycoplasma mycoides* subsp. *capri* in ear swabs taken from goats. Vet. Microbiol. 140, 105–108. doi:10.1016/j.vetmic.2009.06.036

Belloy, L., Janovsky, M., Vilei, E.M., Pilo, P., Giacometti, M., Frey, J., 2003. Molecular epidemiology of *Mycoplasma conjunctivae* in Caprinae: Transmission across species in natural outbreaks. Appl. Environ. Microbiol. 69, 1913–1919.

Besser, T.E., Cassirer, E.F., Potter, K. a., VanderSchalie, J., Fischer, A., Knowles, D.P., Herndon,

247 D.R., Rurangirwa, F.R., Weiser, G.C., Srikumaran, S., 2008. Association of Mycoplasma
248 ovipneumoniae infection with population-limiting respiratory disease in free-ranging Rocky
249 Mountain bighorn sheep (Ovis canadensis canadensis). J. Clin. Microbiol. 46, 423–430.
250 doi:10.1128/JCM.01931-07
251 Cottew, G.S., Yeats, F.R., 1982. Mycoplasmas and mites in the ears of clinically normal goats.
252 Aust. Vet. J. 59, 77–81. doi:10.1111/j.1751-0813.1982.tb02731.x
253 Dagnall, G.J.R., 1993. Experimental infection of the conjunctival sac of lambs with *Mycoplasma*
254 *conjunctivae*. Br. Vet. J. 149, 429–435.
255 DaMassa, A.J., Brooks, D.L., 1991. The external ear canal of goats and other animals as a
256 mycoplasma habitat. Small Rumin. Res. 4, 85–93. doi:10.1016/0921-4488(91)90055-U
257 Degiorgis, M.-P., Frey, J., Nicolet, J., Abdo, E.M., Fatzer, R., Schlatter, Y., Reist, S., Janovsky, M.,
258 Giacometti, M., 2000. An outbreak of infectious keratoconjunctivitis in Alpine chamois
259 (*Rupicapra r. rupicapra*) in Simmental-Gruyères, Switzerland. Schweiz. Arch. Tierheilkd.
260 142, 520–527.
261 Degiorgis, M.-P., Obrecht, E., Ryser, A., Giacometti, M., 1999. The possible role of eye-
262 frequenting flies in the transmission of *Mycoplasma conjunctivae*. Mitteilungen der
263 Schweizerischen Entomol. Gesellschaft 72, 189–194.
264 Fernández-Aguilar, X., Cabezón, O., Frey, J., Velarde, R., Serrano, E., Colom-Cadena, A.,
265 Gelormini, G., Marco, I., Mentaberre, G., Lavin, S., López-Olvera, J.R., 2017a. Long-term
266 dynamics of *Mycoplasma conjunctivae* at the wildlife-livestock interface in the Pyrenees.
267 PLoS One 12, e0186069. doi:10.1371/journal.pone.0186069
268 Fernández-Aguilar, X., Cabezón, O., Granados, J.E., Frey, J., Serrano, E., Velarde, R., Cano-
269 Manuel, F.J., Mentaberre, G., Ráez-Bravo, A., Fandos, P., López-Olvera, J.R., 2017b.
270 Postepizootic persistence of asymptomatic *Mycoplasma conjunctivae* infection in Iberian ibex.
271 Appl. Environ. Microbiol. 83, e00690-17. doi:10.1128/AEM.00690-17
272 Fernández-Aguilar, X., Cabezón, O., Marco, I., Mentaberre, G., Frey, J., Lavín, S., López-Olvera,
273 J.R., 2013. *Mycoplasma conjunctivae* in domestic small ruminants from high mountain
274 habitats in Northern Spain. BMC Vet. Res. 9, 253.
275 Fernández-Aguilar, X., Rossi, L., Cabezón, Ó., Giorgino, A., Llopis, I.V., Frey, J., López-Olvera,
276 J.R., 2017. Infectious keratoconjunctivitis and occurrence of *Mycoplasma conjunctivae* and
277 *Chlamydiaceae* in small domestic ruminants from Central Karakoram, Pakistan. Vet. Rec. 181.
278 doi:10.1136/vr.103948
279 Gelormini, G., Gauthier, D., Vilei, E.M., Crampe, J.-P., Frey, J., Ryser-Degiorgis, M.-P., 2017.
280 Infectious keratoconjunctivitis in wild Caprinae: merging field observations and molecular
281 analyses sheds light on factors shaping outbreak dynamics. BMC Vet. Res. 13, 67–85.

282 doi:10.1186/s12917-017-0972-0
283 Giacometti, M., Janovsky, M., Belloy, L., Frey, J., 2002. Infectious keratoconjunctivitis of ibex,
284 chamois and other Caprinae. Rev. Sci. Tech. 21, 335–45.
285 Glass, H.W., Gerhardt, R.R., 1984. Transmission of *Moraxella bovis* by regurgitation from the Crop
286 of the Face Fly (Diptera: Muscidae). J Eco Entomol 77, 399–401.
287 Gómez-Martín, Á., De la Fe, C., Amores, J., Sánchez, A., Contreras, A., Paterna, A., Buendía, A.J.,
288 Corrales, J.C., 2012. Anatomic location of *Mycoplasma mycoides* subsp. *capri* and
289 *Mycoplasma agalactiae* in naturally infected goat male auricular carriers. Vet. Microbiol. 157,
290 355–362. doi:10.1016/j.vetmic.2012.01.004
291 González-Candela, M., Verbisck-Bucker, G., Martín-Atance, P., Cubero-Pablo, M.J., León-
292 Vizcaíno, L., 2007. Mycoplasmas isolated from Spanish ibex (*Capra pyrenaica hispanica*):
293 frequency and risk factors. Vet. Rec. 161, 167–168. doi:10.1136/vr.161.5.167
294 Maeda, T., Shibahara, T., Kimura, K., Wada, Y., Sato, K., Imada, Y., Ishikawa, Y., Kadota, K.,
295 2003. *Mycoplasma bovis*-associated suppurative otitis media and pneumonia in bull calves. J.
296 Comp. Pathol. 129, 100–110. doi:10.1016/S0021-9975(03)00009-4
297 Mavrot, F., Vilei, E.M., Marreros, N., Signer, C., Frey, J., Ryser-Degiorgis, M.-P., 2012.
298 Occurrence, quantification, and genotyping of *Mycoplasma conjunctivae* in wild Caprinae with
299 and without infectious keratoconjunctivitis. J. Wildl. Dis. 48, 619–31.
300 Mayer, D., Degiorgis, M.P., Meier, W., Nicolet, J., Giacometti, M., 1997. Lesions associated with
301 infectious keratoconjunctivitis in alpine ibex. J. Wildl. Dis. 33, 413–419.
302 Mercier, P., Pellet, M.P., Morignat, E., Calavas, D., Poumarat, F., 2007. Prevalence of
303 mycoplasmas in external ear canal of goats: Influence of the sanitary status of the herd. Small
304 Rumin. Res. 73, 296–299. doi:10.1016/j.smallrumres.2007.01.018
305 Ryser-Degiorgis, M.-P., Ingold, P., Tenhu, H., Tebar Less, A.M., Ryser, A., Giacometti, M., 2002.
306 Encounters between Alpine Ibex, Alpine Chamois and Domestic Sheep in the Swiss Alps.
307 Hystrix 13, 1–11.
308 Ryser-Degiorgis, M.P., Bischof, D.F., Marreros, N., Willisch, C., Signer, C., Filli, F., Brosi, G.,
309 Frey, J., Vilei, E.M., 2009. Detection of *Mycoplasma conjunctivae* in the eyes of healthy, free-
310 ranging Alpine ibex: Possible involvement of Alpine ibex as carriers for the main causing
311 agent of infectious keratoconjunctivitis in wild Caprinae. Vet. Microbiol. 134, 368–374.
312 Tardy, F., Mercier, P., Solsona, M., Saras, E., Poumarat, F., 2007. *Mycoplasma mycoides* subsp.
313 *mycoides* biotype large colony isolates from healthy and diseased goats: Prevalence and
314 typing. Vet. Microbiol. 121, 268–277. doi:10.1016/j.vetmic.2006.12.002
315 Vilei, E.M., Bonvin-Klotz, L., Zimmermann, L., Ryser-Degiorgis, M.-P., Giacometti, M., Frey, J.,
316 2007. Validation and diagnostic efficacy of a TaqMan real-time PCR for the detection of

Mycoplasma conjunctivae in the eyes of infected Caprinae. J. Microbiol. Methods 70, 384–6.

Table 1. Samples and results of *M. conjunctivae* detection in ear, nasal and vaginal swabs shown by species and by detection of *M. conjunctivae* in the eyes. Ruminants were considered positive in the eyes if *M. conjunctivae* was detected in at least one eye.

	Ear		Nose		Vagina	
	Pos/T	**Prev (CI 95%)**	**Pos/T**	**Prev (CI 95%)**	**Pos/T**	**Prev (CI 95%)**
Sheep						
Positive eyes	2/18	11.1 (3.1-32.8)	11/17	64.7 (41.3-82.7)	0/9	0.0 (0.0-29.9)
Negative eyes	4/78	5.1 (2.0-12.5)	1/75	1.3 (0.1-7.2)	0/14	0.0 (0.0-21.5)
Total	6/96	6.3 (2.9-13.0)	12/92	13.0 (7.6-21.4)	0/23	0.0 (0.0-14.3)
Chamois						
Positive eyes	4/9	44.4 (18.9-73.3)	8/12	66.7 (39.1-86.2)	0/1	0.0 (0.0-94.9)
Negative eyes	5/109	3.7 (1.4-9.1)	0/105	0.0 (0.0-3.5)	0/46	0.0 (0.0-7.7)
Total	9/118	6.8 (3.4-12.8)	8/117	6.8 (3.5-12.9)	0/47	0.0 (0.0-7.5)

Iberian ibex						
Positive eyes	2/4	50.0 (15.0-85.0)	1/3	33.3 (1.7-79.2)	NA	NA
Negative eyes	1/42	2.4 (0.1-12.3)	0/25	0.0 (0.0-13.3)	NA	NA
Total	3/46	6.5 (2.2-17.5)	1/28	3.6 (0.2-17.7)	NA	NA
Mouflon						
Positive eyes	1/2	50.0 (2.6-97.4)	2/2	100 (34.2-100.0)	NA	NA
Negative eyes	0/2	0.0 (0.0-65.8)	0/2	0.0 (0.0-65.8)	NA	NA
Total	1/4	25.0 (1.3-70.0)	2/4	50.0 (15.0-85.0)	NA	NA
All species						
Positive eyes	9/33	27.3 (15.1-44.2)	22/34	64.7 (47.9-78.5)	0/10	0.0 (0.0-27.7)
Negative eyes	10/231	4.3 (2.4-7.8)	1/207	0.5 (0.0-2.7)	0/60	0.0 (0.0-6.0)
Total	19/264	7.2 (4.7-11.0)	23/241	9.5 (6.4-13.9)	0/70	0.0 (0.0-5.2)

CI= Confidence Interval; NA=Not Analysed; Pos=Positive; Prev= Prevalence %; T= Total;

340

341 **Table 2.** Results of *Mycoplasma conjunctivae* detection in flying insects. The insects were captured
342 around different ruminant species and infectious keratoconjunctivitis (IKC) epidemiologic
343 scenarios: endemic and asymptomatic infections in sheep flocks and a severe IKC outbreak in
344 captive Iberian ibex.

	Date	Herd Prev. (CI 95%)†	Pos.	Total	Prev. (CI 95%)
Sheep flock 1	10/08/2016	6.7% (1.8-21.3)	0	149	0.0% (0.0-2.5)
Musca spp.			0	146*	-
Stomoxys calcitrans			0	3	-
Sheep flock 2	27/10/2017	0.0% (0.0-13.3)	0	115	0.0% (0.0-3.2)
Musca spp.			0	86*	-
Fannia spp.			0	25*	-
Carabidae spp.			0	2	-
Stomoxys calcitrans			0	1	-
Sarcophaga spp.			0	1	-
Sheep flock 3	31/10/2017	19.4% (9.2-36.3)	(3)	180	1.7% (0.6-4.8)
Musca spp.			(3)*	180*	
Iberian ibex enclosure	26/09/2014	8.7% (3.4-20.3)	2	28	7.1% (2.0-22.6)

Musca spp.		2	28

Pos=Positive; Prev=Prevalence
* Analysed by pools of three specimens.
† Prevalence obtained by ocular detection in any of both eyes.

Fig. 1. Flies spontaneously appear in IKC-affected eyes of Iberian ibex (*Capra pyrenaica*) when taking pictures of the eye lesions. A) Female of *Musca autumnalis* feeding directly from the eye surface. B) High numbers of flies are attracted to the induced eye discharge in IKC-affected animals.

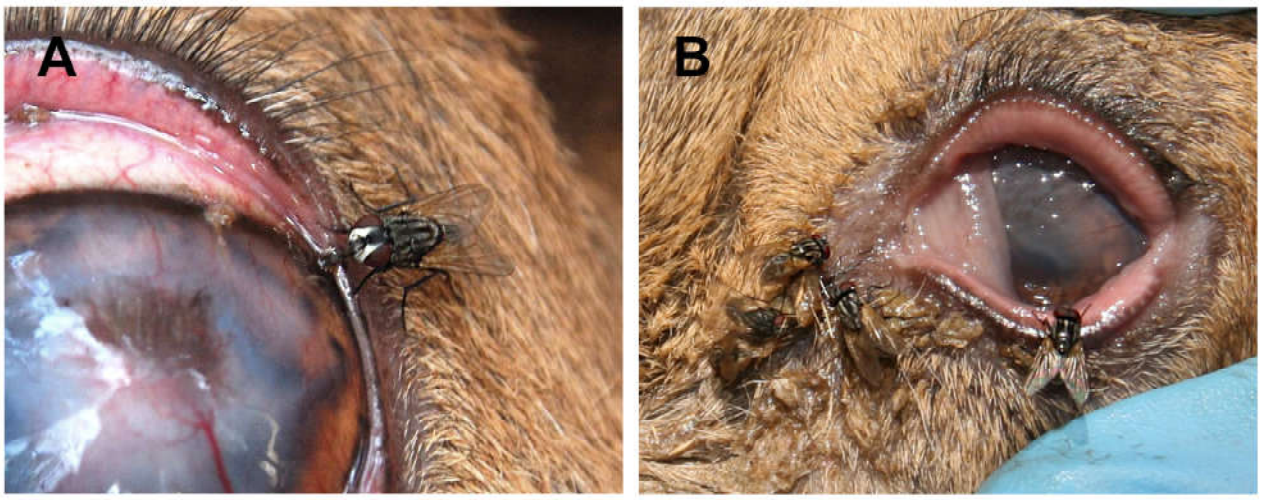
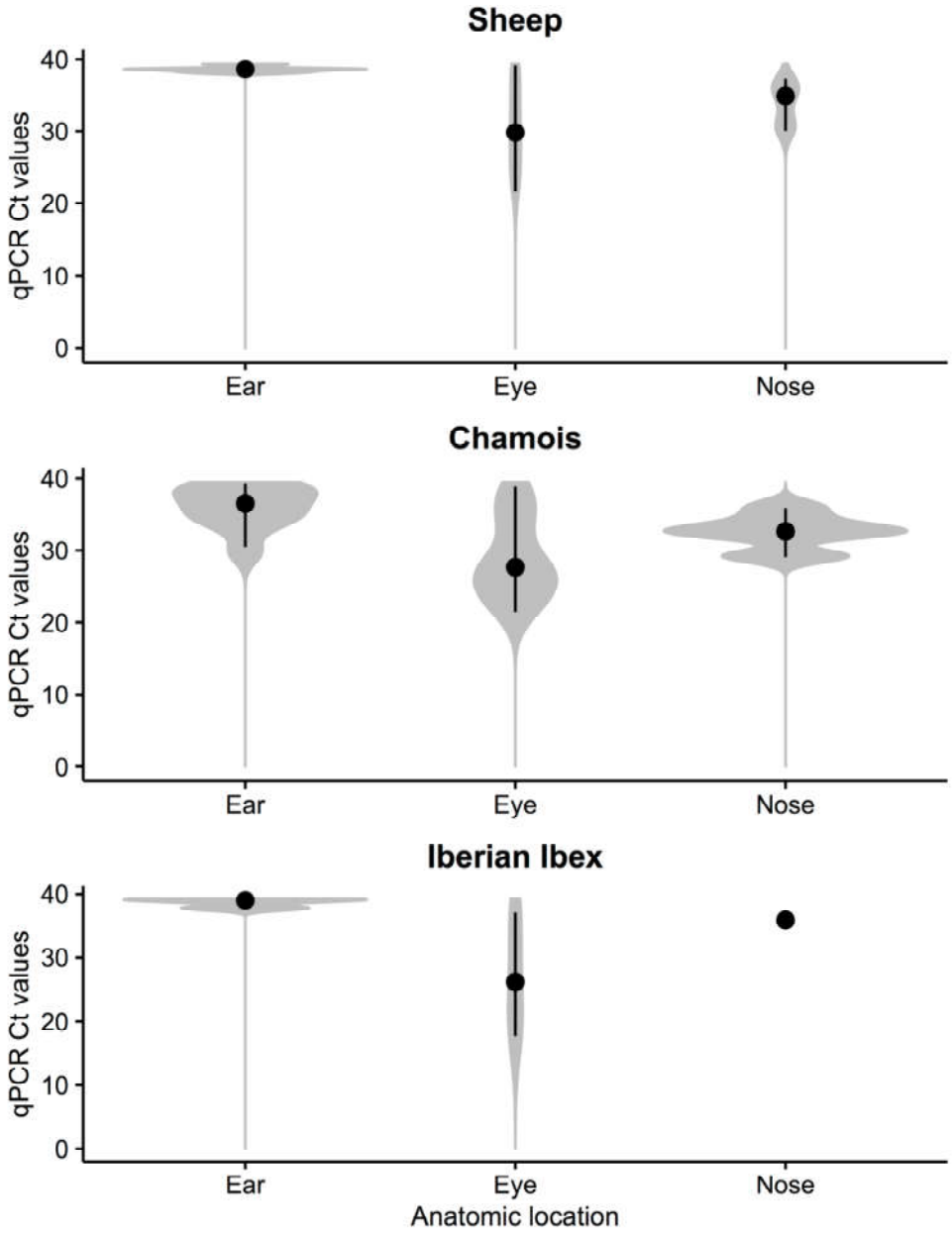


Fig. 2. Violin plots showing the distribution of the threshold cycle values obtained in the qPCR for *M. conjunctivae* detection and the mycoplasma load estimates in ear, eye and nasal swabs. Note that the detection of *M. conjunctivae* in eye swabs was directly performed in cell lysates without DNA extraction and direct comparison of Ct values between nasal and ear swabs is not possible. Black dots indicate the median of the distribution and the bars shows the range of the second and third quartile.


Sheep
qPCR Ct values
40
30
20
10
0
Ear
Eye
Nose
Chamois
qPCR Ct values
40
30
20
10
0
Ear
Eye
Nose
Iberian Ibex
qPCR Ct values
40
30
20
10
0
Ear
Eye
Nose
Anatomic location

356