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

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Highlights

- *Mycoplasma conjunctivae* is detected for the first time in ear canals and flies.
- It was detected in 7.2 % ear and 9.5% nasal swabs from different species.
- Concurrent detection in eye and nasal swabs, but independent to external ear swabs.
- External ear canals may have a role in mycoplasma persistence.
- Mycoplasma was detected in 7.1% *Musca* spp. associated with a disease outbreak.

Abstract

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

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

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

Keywords: chamois; disease transmission; external ear canal; flies; Iberian ibex; Infectious 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,

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

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

2. Materials and Methods

2.1. Study areas and sample collection

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

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

Flying insects from three of the sheep farms sampled and from the Iberian ibex enclosure were captured with a butterfly net and placed in sterile containers. Insects were captured in close proximity/contact with or in the stalls of the animals. The effort of capture performed was similar at each sampling site but differences in flying insect abundance resulted in a different number of specimens captured (Fig. 1). The insects were directly stunned in the freezer and were stored at -20ºC until identification and analysis.

2.2. Sample preparation and M. conjunctivae detection

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

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

The detection of *M. conjunctivae* DNA was performed on the eye swab lysates and the purified DNA from the ear, nose and fly homogenates with a qPCR protocol previously described (Vilei et al., 2007). Briefly, each reaction consisted of 2.5 µl of the sample, 900 nM of LPPS-TM-L primer and LPPS-TM-R, 300 nM of LPPS-TM-FT probe, 12.5 µl of TaqMan®2x Universal PCR MasterMix (Applied Biosystems, Warrington, UK), an exogenous internal positive control (IPC; Applied Biosystems, Warrington, UK) and water up to 25 µl of volume. Positive and negative

controls were included in each plate. The threshold was set at 0.05 and the cycle number when the 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 Ct values lower than 35 in the eyes, approximately 3500 *M. conjunctivae* cells per sample (Vilei et al., 2007), the concurrent detection of *M. conjunctivae* in ear swabs slightly increased to 33.3% (8/24; CI 95% 17.9-53.3), and in nasal swabs increased to 84.0% (21/25; CI 95% 65.3-93.6).

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

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

4. Discussion

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

M. conjunctivae was more frequently detected in the eyes than any other location, supporting its assumed tropism to ocular structures. Our results also suggest that during ocular infections, *M. conjunctivae* commonly colonise EEC in all the species sampled, similarly to that described for other mycoplasmas (Amores et al., 2010; González-Candela et al., 2007). However, the detection of *M. conjunctivae* DNA in EEC was not always associated with its presence in the eyes and indicates the importance of this alternative location for mycoplasma persistence within the host. The longitudinal detection of *M. agalactiae*, *M. mycoides* and *M. capricolum* in EEC during a period of 4 to 10 months supports this hypothesis (Cottew and Yeats, 1982). In this sense, the prevalence of pathogenic mycoplasmas and *M. mycoides* subsp. *mycoides* LC (24%-32%) in the ear canals of goats was also reported to be higher in herds with previous disease history than those without. (Mercier et al., 2007; Tardy et al., 2007). It has been proposed that ear canals have lower immune pressure and thus provide a good niche for mycoplasma persistence (DaMassa and Brooks, 1991). Accordingly, the occurrence of *M. conjunctivae* auricular carriers may contribute to its maintenance in host populations, especially in those where it occurs endemically and with a low eye prevalence (Fernández-Aguilar et al., 2017a; Mavrot et al., 2012).

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

The consistent detection of *M. conjunctivae* DNA among nasal and eye swabs and the positive correlation of Ct values of these two sites suggests that the nasal cavity is most probably an excretion route through the natural anatomic communication with the eyes, but not a location for the persistence of the mycoplasma. According to the relatively low Ct values detected in nasal swabs, nasal secretions are probably an important source for *M. conjunctivae* transmission. This is

in agreement with the early reports of *M. conjunctivae* in nasal smears of sheep, and the high loads—up to 10^7 CFU/ml—isolated from the nasopharynx in experimentally infected sheep (Dagnall, 1993). However, direct comparison of the Ct values between eye and nasal swabs can not be performed because of the different methods used for the molecular detection.

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

Insights on the diversity of eye- and carcass-frequenting insects in wild and domestic ruminants indicated that up to four former genera of Muscidae (*Hydrotaea*, *Musca*, *Morellia* and *Polietes*) might be involved in IKC epidemiology in the Swiss Alps (Degiorgis et al., 1999). In the present study, we found lower diversity but also different genera of Diptera, among which *M. conjunctivae* was only detected in *Musca* spp. Some specimens not included in this study but captured at the same time and locations were identified as *Musca domestica* (sheep flocks) and *Musca autmnalis*

(Iberian ibex enclosure). The feeding habits of *Musca* spp. on lacrimal and other body secretions (Glass and Gerhardt, 1984) strongly suggests that this genus of flies are relevant vectors for IKC in Caprinae.

Further assessment of the viability of *M. conjunctivae* cells should be performed to confirm the 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.

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Declarations of interest: None.

Conflict of interest statement

The authors declare no conflicts of interest.

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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.

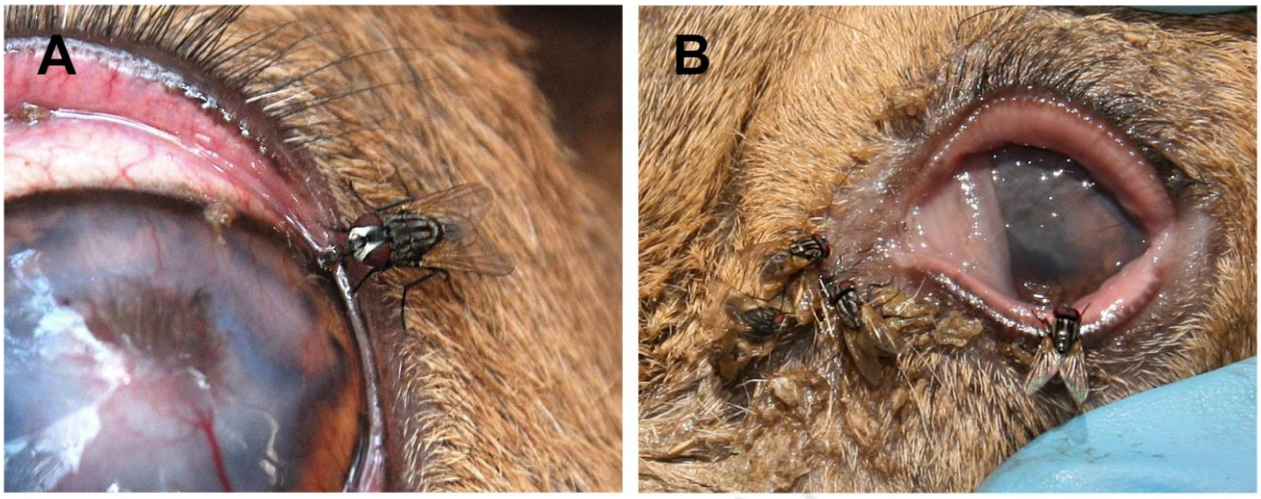
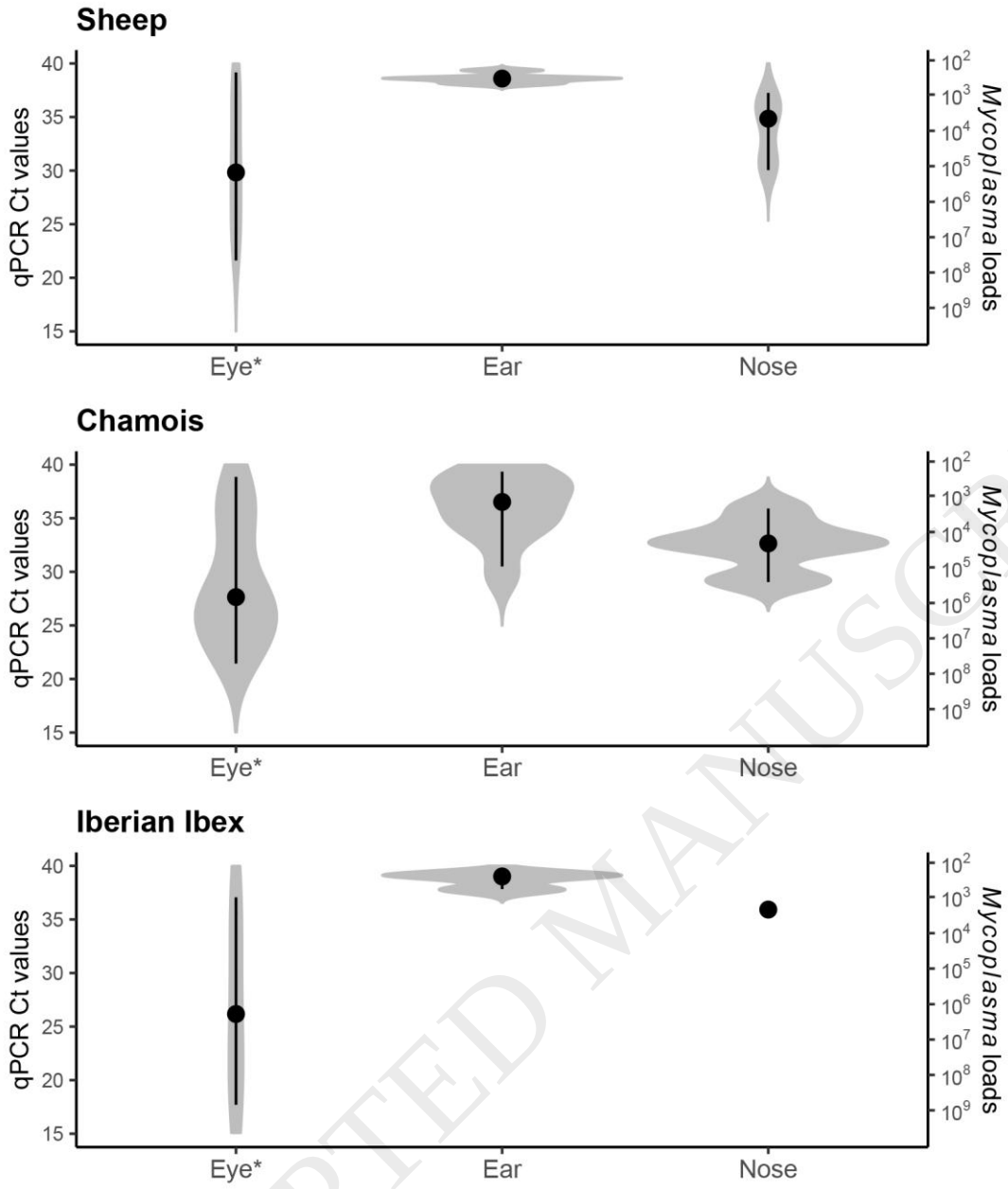


Fig. 2. Violin plots showing the distribution of the threshold cycle values obtained in the qPCR for *M. conjunctivae* detection and the mycoplasma load estimates in ear, eye and nasal swabs. Note that the detection of *M. conjunctivae* in eye swabs was directly performed in cell lysates without DNA 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.



*Detection from cell lysates

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;

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