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Abstract

The sheep ked, *Melophagus ovinus*, and the forest fly, *Hippobosca equina*, are parasitic dipteran insects of veterinary importance. As hematophagous insects, they might be considered as potential vectors of diseases which may be transmissible to humans and animals. The purpose of this study was to present initial primary data about these two species in Algeria. To do so, we conducted a molecular survey to detect the presence of bacterial DNA in flies collected in Algeria. A total of 712 flies including, 683 *Melophagus ovinus* and 29 *Hippobosca equina* were collected from two regions in northeastern Algeria. Monitoring the monthly kinetics of *M. ovinus* infestations showed something resembling annual activity, with a high prevalence in January (21.67%) and May (20.94%).

Real-time quantitative PCR assays showed that for 311 tested flies, 126 were positive for the *Bartonella* spp. rRNA intergenic spacer gene and 77 were positive for *Anaplasmataceae*. A random selection of positive samples was submitted for sequencing. The DNA of *Bartonella chomelii* and *Bartonella melophagi* were amplified in, respectively, five and four *H. equina*. 25 *M. ovinus* positive samples were infected by *Bartonella melophagi*. Amplification and sequencing of the *Anaplasma* spp. 23S rRNA gene revealed that both species were infected by *Wolbachia* sp. which had previously been detected in *Cimex lectularius* bed bugs.

Overall, this study expanded knowledge about bacteria present in parasitic flies of domestic animals in Algeria.

Keywords: Sheep, *Melophagus ovinus*, Horses, *Hippobosca equina*, vector-borne diseases, Algeria.

1. Introduction

Hippoboscidae flies, usually known as keds or louse flies, are obligate hematophagous Diptera, which bite birds and some mammals **[1, 2]**. They are organised into more than 19 genera and 150 cosmopolitan species **[3-6]**. Members of this family, particularly those relating to sheep (*Melophagus ovinus*), horses (*Hippobosca equina*) and dogs (*Hippobosca longipennis*) bite their hosts and people who take care of these animals **[1, 3, 7]**. Human reactions to these bites vary widely, ranging from simple redness followed by pruritic inflammation to anaphylactic shock requiring emergency treatment **[8-11]**. In animals, these ectoparasites are responsible for weight loss, decrease in wool growth and livestock milk production, and cutaneous myiasis causing, in the long run, significant economic losses **[1, 7, 12-14]**.

The pathogenic role of Hippoboscidae flies remains insufficiently documented. As they are often subservient to the hosts they parasitize, this could cast doubt on their ability to transmit pathogens to other animals or humans **[3]**. However, recent studies have reported the molecular detection of several vector-borne pathogens in Hippoboscidae flies collected on ruminants **[15-22]**, horses **[15, 23]**, dogs **[24, 25]** and raptors **[26]**.. All these studies support the hypothesis that Hippoboscidae flies might be vectors of infectious diseases. However, molecular studies are not sufficient to confirm the vector competence of an arthropod.

Although *M. ovinus* is the most studied Hippoboscidae alongside *H. equina* **[1, 27, 28]**, data on these two species in terms of their biology and involvement in the transmission of pathogens are entirely lacking in Algeria. The main reason for this is that *M. ovinus* and *H. equina* flies often go unnoticed and are considered by farmers to be harmless pests.

It was from this perspective that we investigated Hippoboscidae flies that parasitize sheep and horses in northeastern Algeria. The aims of the present study were to gather initial data on the monthly prevalence of *M. ovinus* and high-risk periods, and to use molecular procedures to investigate the presence of bacteria in *M. ovinus* sheep keds and *H. equina* forest flies.

2. Materials and Methods

2.1. Study areas and period of collection

The study was carried out between August 2015 and July 2016 in two regions of the extreme north east of Algeria: El Tarf (36°51'21.5"N, 8°19'34.5"E) and Mila (36°27′0″N, 6°16′0″E) **(Fig. 1)**. These regions are known for cattle breeding and have fairly similar climates. El Tarf is made up of two clearly differentiated areas: the northern part is mainly characterised by alluvial plains and the climate is sub-humid to warm humid; while the southern part has greater relief and the climate is humid to cool and wet **[29]**. The Mila region is notable for its humid climate in the north, sub-humid to semi-arid in the centre, and semi-arid in the south **[29]**.

2.2. Fly collection and identification

Melophagus ovinus flies were collected on a monthly basis from three sheep farms located in the town of Tessala Lematai in the Mila region. These farms culminate at approximately the same altitude (~ 1,465m) and are each composed of 30 sheep of different sex and age categories. For every ovine which was examined, its age (young: ≤ 1 year; adult: >1 year) and sex were recorded. To collect the flies, the fleeces of the parasitized sheep were parted and the flies were collected directly from the deepest parts of the wool. During the shearing period in May, the flies were recovered directly and individually from the wool bales placed beside each sheared sheep. In both cases, sheep keds were carefully removed and immediately placed in 70% ethanol. The *M. ovinus* pupae were not recovered from infested animals and were left *in situ*.

The *Hippobosca equina* forest flies were collected only once, in June, from two horse barns in Ain El Kerma, a town in the El Tarf region. The first barn consisted of 11 horses while the second was composed of nine horses. The flies were caught manually from the inner thigh and around the perineum of parasitized horses and were directly stored in 70% ethanol.

The sampled flies were morphologically identified using a Leica® binocular lens with an LED light at the IHU Méditerranée Infection, Marseille, France. Identification was essentially based on the morphotaxonomic criteria reported in Huston and Wall and Shearer dichotomous keys **[30, 31]**. Photographs of the dorsal and ventral sides of each species were taken with a microscope at a magnification of ×56 (Zeiss Axio Zoom.V16, Zeiss, Marly le Roi, France) **(Fig. 2 and 3)**.

2.3. DNA extraction

DNA extraction was performed on a representative selection of *M. ovinus* flies from each of the three sheep farms and on all the *H. equina* fly specimens. All experiments and sample handling was conducted under sterile conditions under a laminar flow biosafety hood. The sample preparation process was the same for both fly species. The flies were removed from the ethanol, rinsed for 10 minutes in a sterile distilled water bath and then dried with filter paper. For each sample, a longitudinal incision was made using a scalpel blade, cutting the fly into two equal parts. One half was dropped into a sterile tube (Eppendorf; Hamburg, Germany) while the remaining part was kept at -20°C for further analysis.

Each half-fly was then incubated at 56°C overnight with 180µL of G2 lysis buffer (Qiagen, Hilden, Germany) and 20µL proteinase K (Qiagen, Hilden, Germany). DNA extraction was processed using an EZ1® DNA Tissue Kit (Qiagen, Hilden, Germany) and EZ1® BioRobot® extraction device according to the manufacturer's recommendations. Between each batch, all parts of the device were disinfected and subjected to 20 minutes of ultraviolet light to avoid any cross contamination. Finally, the DNA from each sample was eluted in 100 µL of Tris-EDTA (TE) buffer (Qiagen, Hilden, Germany) and stored at -20°C under sterile conditions.

2.4. Molecular survey and PCR amplification

To investigate the presence of bacterial DNA, all the extracted DNA was subjected to real-time PCR assay using a CFX ConnectTM Real-Time PCR Detection System (Bio-Rad, Marne la Coquette, France) targeting a fragment of specific genes of five bacteria. The citrate synthase (*gltA*) "RKND03" gene was used to detect *Rickettsia* spp. **[32]**, the intergenic spacer (ITS2) gene was used to detect *Bartonella* spp. **[33]**, the 23S ribosomal RNA gene was used to detect the *Anaplasmataceae* bacteria **[34]**, the ITS4 spacer was used to detect *Borrelia* spp. **[35],** and IS30A spacers were used to detect *Coxiella burnetii* **[36]**.

15µl of the qPCR reaction mix, without any DNA, and dilutions of DNA extracts of cultured bacteria strains were used in each test respectively as negative and positive controls, as previously described **[37]**. Samples were considered positive when the cycle threshold (Ct) value given by the Bio-Rad CFX Manager™ v3.1 software (Bio-Rad, Marne la Coquette, France) was ≤35. A random selection of these positive samples was subsequently subject to conventional PCR prior to sequencing in order to identify pathogens at the species level.

Conventional PCR analysis was performed using an automated DNA thermal cycler (Applied Biosystems, 2720, Foster city, USA), targeting the intergenic transcribed (ITS) gene **[38]** and the citrate synthase (gltA) gene **[39]** for qPCR *Bartonella* spp. positive samples. Bacterial DNA from the Anaplasmataceae family was detected using the 23S gene **[40]**. Amplified products were then subjected to electrophoresis through a 0.5% agarose gel stained with SYBR Safe™ and viewed using a ChemiDoc™ MP ultraviolet imager (Bio-Rad, Marnes-la-Coquette, France).

The PCR products were purified using a Macherey-Nagel plate (NucleoFast® 96 PCR, Düren, Germany), as recommended by the manufacturer. Amplicons sequences were obtained using the ABI Prism 3130xl (ABI PRISM, PE Applied Biosystems, USA) genetic analyser

capillary sequencer and the BigDye® Terminator v1.1, v3.1. 5x Sequencing Buffer (Applied Biosystems, Warrington, United Kingdom).

2.5. Sequence processing

In order to identify bacterial species, the obtained nucleotide sequences were first assembled and edited using ChromasPro v.1.7.7 software (Technelyium Pty. Ltd., Tewantin, Queensland, Australia). They were then processed by comparing them with the sequences available in the GenBank database, using the online Basic Local Alignment Search Tool (BLAST) (http:// blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. Statistical analysis

Statistical analyses were performed using the SPSS v24.0 HF02 software (IBM SPSS Statistics for Windows, Version 24.0, Armonk, NY: IBM Corp, 2016), and the Pearson's chi-squared "$\chi 2$" test was used to compare the overall prevalence of *M. ovinus* according to the month of collection and the age and sex of the sheep.

2.7. Ethical considerations

Ethical consent for sampling from sheep and horses was granted by the El Tarf University Animal Ethics Committee. Verbal agreement for the field study was obtained from the animals' owners and from the local agricultural services office.

3. Results

3.1. Fly collection and infestation prevalence

A total of 683 adult *M. ovinus* flies (320 males and 363 females) and 29 *H. equina* flies (14 males and 15 females) were collected respectively from 81/90 (90%) infested sheep belonging to the three Mila farms and 9/20 (45%) infested horses from the El Tarf barns.

The sheep studied here were also co-infested by other ectoparasites such as *Damalinia ovis* lice, and *Haemaphysalis sulcata* and *Haemaphysalis punctata* ticks, while the horses were infested by *Damalinia equi* lice and *Boophilus (Rhipicephalus) annulatus* ticks.

Monitoring of the monthly kinetics of *M. ovinus* infestation in the three sheep farms showed that these flies parasitize animals almost all year round, with a high prevalence in January (21.67%) and May (20.94%) and almost zero prevalence in autumn and summer **(Fig. 4)**. These results allow us to define a statistically significant ($\chi 2$ test, $\rho = 0.01$) period of activity for this fly species which can be estimated to exist between the months of October and May.

In addition, we compared the ovine infestation rate by the age and sex of the sheep. The results revealed that there was no statistically significant difference in *M. ovinus* infestation rates between male and female sheep ($\chi 2$ test, $\rho = 0.58$) and between young and adult sheep ($\chi 2$ test, $\rho = 0.21$).

The horses' infestation by the *H. equina* forest fly could not be correctly monitored due to the frequent sales of the horses, which resulted in off-peak periods where no horses were present in the stables.

3.2. Detection of bacteria

From 712 collected flies, 311/712 specimens including, 282 *M. ovinus* (94 specimens from each of the three farms) and 29 *H. equina* were randomly selected and screened using real-time PCR for the presence of bacteria. Of the 311 flies tested, 202/311 (64.95%) samples were positive for at least one of the investigated bacteria, while 58/311 (18.64%) were found

to be co-infected by two bacterial genus. The 311 flies tested negative for all spotted fever group *Rickettsia* species, *Borrelia* spp., and *C. burnetii*. The bacterial identity of a random selection of the positive samples was later achieved by standard PCR amplification and sequencing.

Real-time assays targeting the intergenic spacer (ITS) gene for *Bartonella* spp. revealed that 126/311 (40.51%) flies, including 104/282 (36.87%) *M. ovinus* and 22/29 (75.86%) *H. equina* collected respectively from sheep and horses, were *Bartonella*-positive. From 126 *Bartonella*-positive samples, all 22/29 *H. equina* specimens and 25/104 randomly selected *M. ovinus* specimens were subject to standard PCR. However, amplification trials using the ITS gene were not successful. Sequencing analysis using the *gltA* system was subsequently performed and high quality sequences were obtained for all (25/25) *M. ovinus* specimens and 9/22 of the *H. equina* flies. The BLAST analysis results revealed 99.48 to 100% identity with the corresponding 753 base pair fragment of *Bartonella chomelii* (GenBank accession nos. KM215691.1 and KM215693.1) for 5/9 sequences amplified from *H. equina* and 100% identity with *Bartonella melophagi* (GenBank accession nos. MG701237.1) for the remaining sequences (4/9). However, following the BLAST query, all sequences obtained from *M. ovinus* specimens showed 99.50 to 100% identity with the corresponding 323 base pair fragment of *B. melophagi* (GenBank accession nos. MG701237.1).

Of the total 23S qPCR screened flies, 77/311 samples (24.75%) involving 73/282 *M. ovinus* (25.88%) and 4/29 *H. equina* (13.79%) were infected by bacteria from the *Anaplasmataceae* family. Within this, 23S rRNA amplification and sequencing was successful for 65/77 samples (61 *M. ovinus* and four *H. equina*). The query of the resulting sequences against the NCBI GenBank database showed, for both fly species, 99.31 to 100% identity with the "*Wolbachia endosymbiont of Cimex lectularius"* sequence (GenBank accession nos. AP013028.1).

4. Discussion

Over last decade, the vast majority of research conducted on parasitic arthropods of animals in Algeria has essentially focused on ticks, fleas, mosquitoes, and sand-flies. The interest in these categories of pests largely reflects the fact that they can significantly affect their hosts by transporting and inoculating several vector-borne diseases **[37, 41-45],** which can in turn lead to considerable economic losses.

To date, few entomological investigations into haematophagous flies and their associated microorganisms have been the subject of research in Algeria. The only available records were inventories on *Nycteribiidae* bat flies, which may be recognized as potential vectors of *Bartonella tamiae* **[46, 47]**. The unpredictable behaviour of the Hippoboscidae fly its voracity and its spectacular ability to reproduce and quickly increase the number of individuals per population, as well as and its ability to cover long distances in flight, has significantly increased the vulnerability of animals to vector-borne pathogens. Consequently, the fly-bacteria relationship should not be overlooked.

In this study, we have observed the prevalence and the period of occurrence of the *M. ovinus* sheep ked by conducting an annual monitoring of the infestation. An overall prevalence of 90% for *M. ovinus* registered in our study can be explained by the combination of various crucial factors including the presence of a cold climate and a high altitude region which are absolutely essential to the fulfilment of *M. ovinus* life cycle **[1, 48]**; The mismanagement of the livestock and unhealthy husbandry conditions which could predispose malnourished sheep to infestation **[49]**; and finally the highly abusive use of insecticides which could lead to the emergence of a resistant cohort of keds **[12]**. In addition, the annual monitoring of *M. ovinus* population revealed that the number of sheep keds began to increase in late October, peaked in January and May and observed a seasonal low starting from late May. These findings are in

compliance with previous observations where *M. ovinus* populations are reported to be active in winter and spring and less in summer **[50, 51]**.

On the other side, the lack of a statistically significant difference in infestation rates of *M. ovinus* depending on the sex and ages of infested sheep is consistent with previous studies where the abundance of *M. ovinus* flies in sheep was shown to be related to abiotic components rather than host dependent factors **[1, 49]**.

Molecular tools were used to conduct the bacteriological survey. The reliability of these techniques in such epidemiological investigations on arthropods and especially on flies has been demonstrated in previous research **[15, 16, 20, 49]**. Furthermore, the results reported here were obtained under sterile conditions using meticulous laboratory procedures and sophisticated instruments routinely employed in our laboratory. Although a negative and positive control of each PCR test was used to confirm the accuracy of our results, this cannot confirm the vector role of the flies studied, as they could have acquired the bacteria after blood meals on infected animals or crossed other vectors and co-fed with them.

This survey demonstrated that 64.95% of tested flies were infected by at least one bacteria. *B. melophagi* was found in *M. ovinus* and *H. equina* while *B. chomelii* was detected in *H. equina*. Phylogenetically, these two bacteria are affiliated, alongside *B. bovis, B. capreoli* and *B. schoenbuchensis,* to *Bartonella* strains which mainly infect ruminants **[52]**. However, their pathogenicity and the way that they may be transmitted are poorly understood.

Nevertheless, the manifestation of symptoms in patients who are in contact with animals and who suffered from idiopathic pericarditis, circular red skin lesions, and chronic asthenia have been associated with the clinical expression of a *B. melophagi* infection which was later isolated from their blood **[11]**. Although sheep were reported as the most likely reservoirs of *B. melophagi* **[11, 53, 54]** there is no symptomatic description of such infections in animals.

However, as certain *Bartonella* species were associated with bovine endocarditis **[55, 56]** further investigation of *B. melophagi* are needed.

Bartonella chomelii has not yet been shown to be pathogenic **[57-59]**. This *Bartonella* species was first isolated from domestic cattle blood **[60]** and was later identified as the most common *Bartonella* infecting cattle **[61]**.

Both species of *Bartonella* adapted to mammal reservoirs and, according to previous studies, they share biting flies as common potential vectors **[54]**. This symbiosis is possible when a variety of adaptation, metabolic interconnections and genomic changes occur between insects and bacteria.

The occurrence of *B. melophagi* within *M. ovinus* sheep keds has already been reported in previous works **[21, 49, 54, 62, 63]** and *B. melophagi* DNA was found in the gut of *M. ovinus* adults and even in the pupal stages, thus suggesting this bacteria is endosymbiotic in nature, and confirming our findings **[15, 21, 49, 62, 64, 65]**. However, *B. melophagi* had never been detected in *H. equina*. To the best of our knowledge, this is the first report of such an association. The few available reports until now have associated *H. equina* flies with *B. schoenbuchensis* and *B. chomelii* **[15, 23]**.

Bartonella chomelii has been associated with several arthropods, such as Ixodid ticks and biting flies **[15, 66, 67]**. However, its most effective vector remains unknown.

Overall, the flies sampled in this survey were infected by two *Bartonella* species and, as alluded to above, this does not guarantee their role as vectors. Further investigations are needed to shed light on the role of *H. equina* in the epidemiology of *B. melophagi* and *B. chomelii*.

This study also investigated the presence of bacteria from the Anaplasmataceae family. The ability of hippoboscidae flies to vector bacteria from this family has been recently assessed. The results confirmed the presence of *Anaplasma ovis* in *M. ovinus* specimens and its vertical

transmission among keds **[16, 18],** thus bringing new evidence regarding the potential position of these flies as mechanical or biological vectors. In our case, "*Wolbachia endosymbiont of Cimex lectularius"* was detected in 61 *M. ovinus* and four *H. equina*. To the best of our knowledge, this study provides the first molecular proof of the presence of *Wolbachia* spp. DNA in *H. equina* adults. However, these bacteria have already been described as the third most common bacteria genus after *Bartonella* and *Arsenophonus* in the midgut of *M. ovinus* **[63, 64]**. In addition, the screening of *M. ovinus* microbiota revealed that *Wolbachia* was present intracellularly in various tissues such as adipocytes, secretory cells and intestinal tissue **[65]**. These multiple locations of *Wolbachia* spp. have previously been described for other arthropods such as lice and bed bugs **[68]**. However, no studies have so far reported the presence of *Wolbachia* spp. or *Anaplasma* spp. in *H. equina* flies.

5. Conclusion

In this study, we provided molecular evidence for the presence of bacteria in two fly species collected from sheep and horses in northeastern Algeria. *B. chomelii* has been detected for the first time in Algeria and the African continent, while *B. melophagi* is reported for the first time in Algeria. In view of these facts, more attention should be given to livestock haematophagous flies, since they can carry zoonotic pathogens. Measures to combat them could be conducted according to high-risk periods. However, the choice of the most effective insecticide will have to be made according to two parameters: its long persistence and its quality/price ratio.

Competing interests

The authors declared that they have no competing interests.

Author contributions

MB contributed to arthropod collection, performed DNA extractions, PCRs, sequencing and prepared the first draft of the paper. **NM** helped with fly sampling and contributed to the manuscript. **AB** contributed to conceiving, designing and coordinating the study. **DR** contributed reagents/materials/analysis tools. **PP*** coordinated experiments and reviewed the paper.

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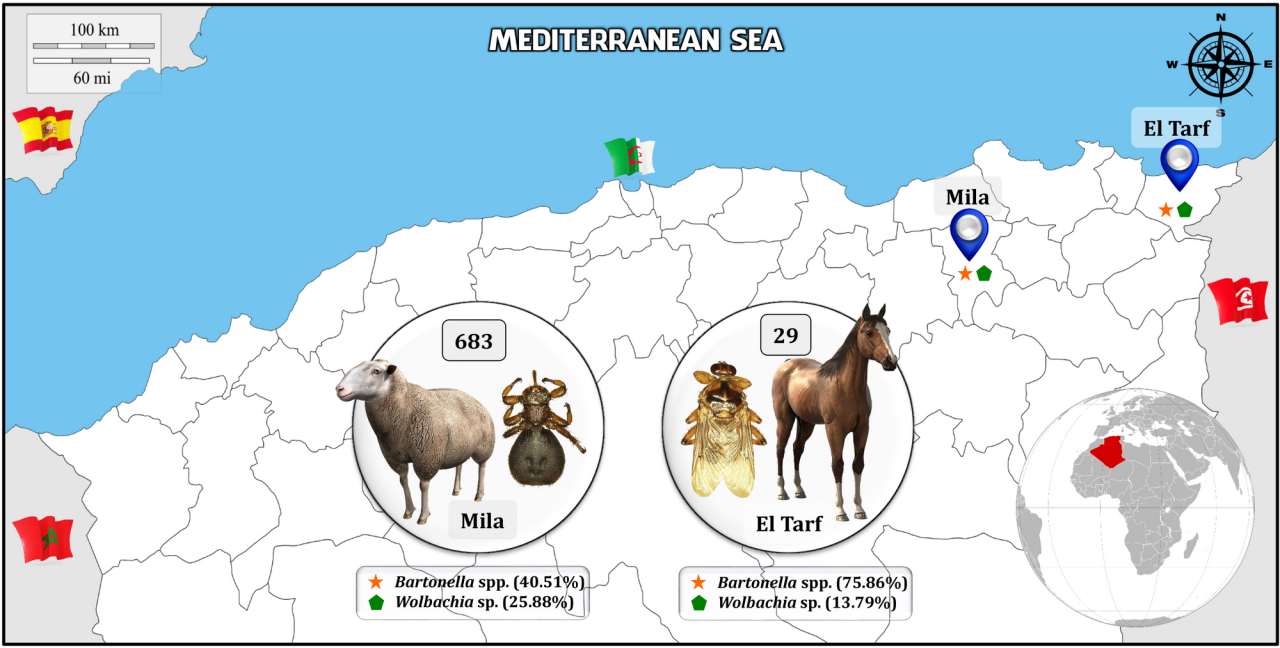
Figure legends

Fig. 1: Collection sites of *Melophagus ovinus* and *Hippobosca equina* and their infection rates by *Bartonella* spp. and *Wolbachia* sp.

Fig. 2: The "false sheep louse" *Melophagus ovinus* (Diptera: Hippoboscidae): A 4-7 mm fly with wings which have been reduced to scales and no halters and a reddish-brown body covered by dense setae. The head is embedded in the thorax with reduced compound eyes; the abdomen is heart-shaped (narrow in the male, wider in the female). Three pairs of legs each ending in large claws. The mouthparts consist of a prominent piercing-sucking proboscis. Male: Dorsal **[a]** and ventral view **[b]**; Posterior end **[c]**; Pupae are progressively coloured and tanned from light (right) to dark brown (left) **[d]**. Female: Dorsal **[e]** and ventral view **[f]**; Posterior end **[g]**.

Fig. 3: The "forest fly" *Hippobosca equina* (Diptera: Hippoboscidae), 7-8 mm, horse, cattle and dromedary parasite. The wings are large and longer than the body and slightly tinted, with seven longitudinal veins and two cross-veins **[h]**. The body is reddish brown with yellow bands and dense setae, wide and flat thorax and compound big eyes. Three pairs of legs each ending in strong claws. Moves sideways, like a crab; fast flight. Male: Dorsal **[i]** and ventral view **[j]**; Posterior end **[k]**. Female: Dorsal **[l]** and ventral view **[m]**; Posterior end **[n]**.

Fig. 4: Overall monthly prevalence of melophagosis in three sheep farms in Mila.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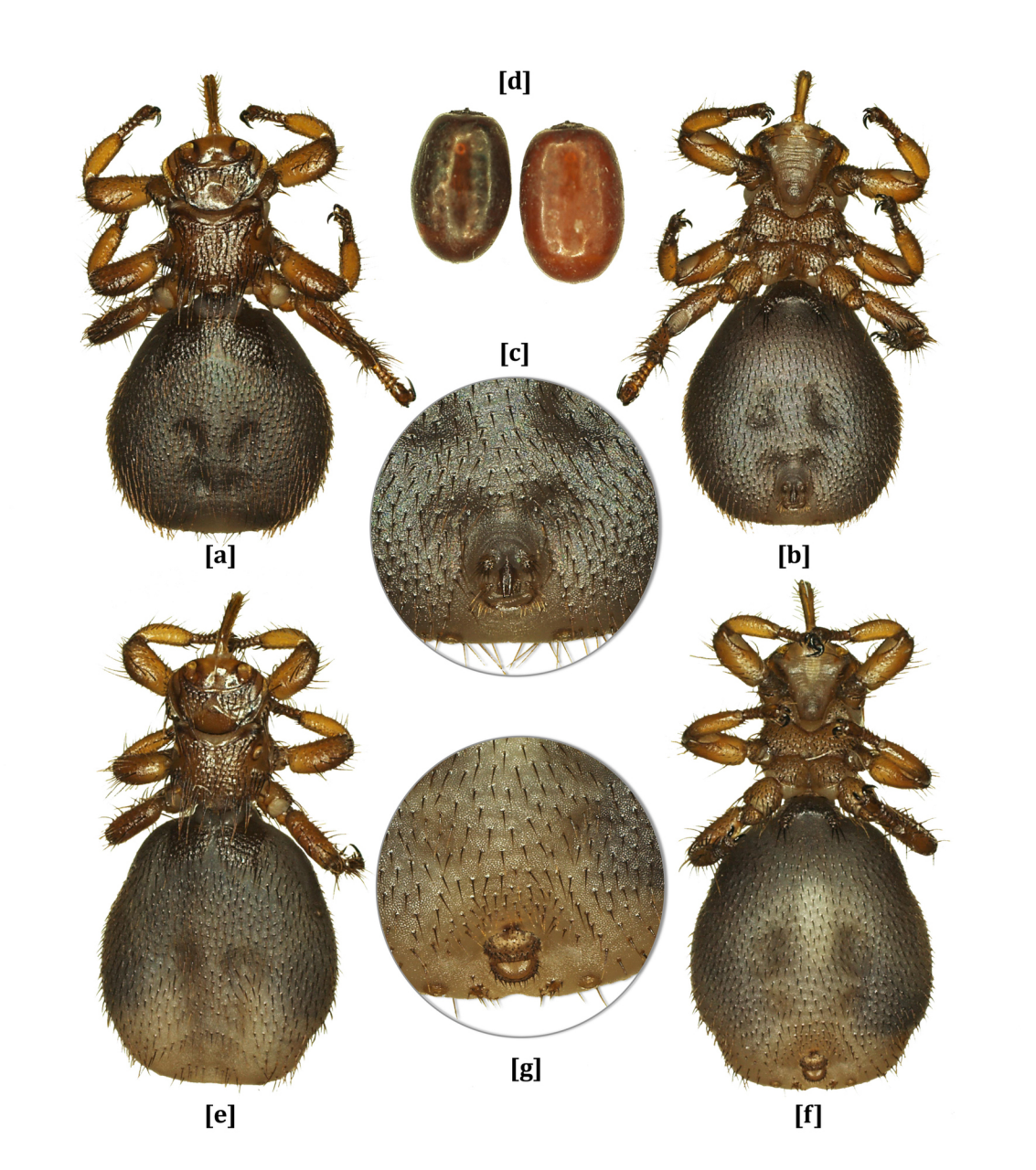
Bartonella spp. (75.86%)
Wolbachia sp. (13.79%)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