Response to Hélène Jourdan

General comments:
This is a really nice study that investigate the role of the soft tick, *Ornithodoros maritimus*, as a potential vector of *Babesia* sp. YLG. This study relies on tick sampling during 4 successive years on the islet Carteau which constitutes a simplified environment with only one vertebrate host and one tick species. Analyses are based on tick dissection to isolate different organs involved in pathogen transmission (salivary glands and ovaries) and test for the presence of Babesia DNA. The molecular tests also involved a control of potential contamination of tick organs by potentially infected host blood present in the caecum. This is a nice study, well written and thus easy to read. One of my major concern, which will be easily corrected, is the absence of any statistical test, despite mentions of differences in infection rates. Some clarifications are also needed throughout the manuscript.
Once these issues are addressed, I believe that this preprint can be recommended in PCI Infections.

Specific comments:

-L110: "*nymphs had undergone metamorphosis before they were analyzed* ». I believe that this means larvae having molted into nymphs. It sounds as it nymphs were able to undergo a molt without blood feeding. Please clarify.

REPLY: Thank you for this constructive comment, we realized that our text could be confusing. Some of the collected nymphs were engorged and moulted before arriving in the lab. We changed the text to: "In some cases, engorged ticks laid eggs (if female) or moulted into subsequent life stages prior to analyses. Freshly moulted nymphs and females were used to study trans-stadial transmission of the parasites and eggs to study the transovarial transmission." Lines 104-107. We hope this is clearer.

-L121 : Figure1 : « *C : Caeca* ». I think that this is misleading to use C here to abbreviate Caecum since C is also the name of one picture with salivary glands.

REPLY: We agree about the possible confusion. We therefore modified the abbreviation of caeca from C to Ca in the Figure 1 as well as in Table 2 and in the text.

-L 148-149 : Is it a nested PCR as for the amplification of 18S from YLG ?

REPLY: We didn't need the sensitivity of a nested PCR for the amplification of *O. maritimus* 18S rDNA. For clarity, we changed "template DNA" to "total genomic DNA" (line 146).

-L180-180 : How were ticks chosen since you mention that they represent only a small fraction of the ticks that were present in sampled nests ? Randomly ? Preferentially females ?

REPLY: A random selection of ticks that we sampled from each nest were used for analyses. We know from experience that our sampling scheme (standardized 6min search of nest material in situ), only represents a small fraction of the ticks that are actually present in the nest area (see Dupraz et al. 2017 IPJW). We have clarified this part of the text to provide more direct information on the sampling scheme (lines 183-187):

"All ticks were found in active nests during standardised searches (see Dupraz et al. 2017). Most were females (107), but males (15) and nymphs (22) were also sampled (Fig. 2). Four females and eight nymphs were freshly moulted. These ticks came from more than 38 different nest sites (6 in 2019, 26 in 2020, 6 in 2021, unknown for 2022), and represent a random sample of ticks from each sampled nest".

L181 : How do you define active nests ?

REPLY: Active nests mean nests with YLG eggs or young chicks.

L194 : « *The 18S rRNA gene of O. maritimus was detected in all DNA extracts.* » Which DNA extracts are they ? extract from all organs ? Caecum ?

REPLY: We are referring here to all DNA extracted from tick organs. To clarify, we have changed the text to: "in all DNA extracts from tick organs." (line 195).

L196 : «*26.1% in 2019 (6/23 tested ticks), 15.9% in 2020 (10/63 tested ticks), 39.5% in 2021 (15/38*
197 tested ticks) and 45% in 2022 (9/20 tested ticks) ». Is the infection rate significantly different between years ?

REPLY: We didn't initially perform statistical tests from ticks collected in consecutive years as to the aim here was to check the persistence of infection in the colony. We have provided the results of a Fisher's Exact test and modified the text to clarify this point (line 199).

L 197 : « *All tick stages were found infected, females (31.8%, 198 34/107), males (26.7%, 4/15) and nymphs (9.1%, 2/22).* » Is it significantly different ?

REPLY: The results of a Fisher's exact test have been added to the text (line 202).

L198-199 « *Positive ticks came from 18/38 of the identified ~~199~~ sampled nests (4/6 in 2019, 9/17 in 2020, 5/6 in 2021, unknown in 2022)* ». I don't understand how you can compute this ratio since the nest origin was not recorded. I see that you sumed the number of positive nests in 2019, 2020 and 2021 over (I guess) a total number of nests sampled in 2019, 2020, 2021 and 2022. This does not seem the right way to do it. You could calculate this ratio over 2019, 2020 and 2021, excluding 2022.

REPLY: This is a good suggestion. We made an error in the number of nests in 2020 and corrected it (line 203).

L206-207 : « *Prevalence of positive salivary glands tended to vary among life stages: females (15/103, 14.6%), males 207 (1/12, 8.3%) and nymphs (1/22, 4.5%).* » Did you test the statistical differences between stages ? For example you could simply use **prop.test** in R

REPLY: The results of a Fisher's exact test have been added to the text (line 210).

L 209-210 « *However, for three, Babesia sp. YLG DNA was not found in the gut content, suggesting that the salivary glands were truly infected and the positive result was not due to a contamination by infected gut content*. » I don't understand the difference with the 17

salivary glands mentioned before (L204) where the parasite was detected without traces of gut contamination.

REPLY: As these details seem to be confusing and do not change the main result, we propose to simply delete this section to improve clarity.

L 215 Figure 3 : « *F : newly moulted females* ». They are not mentioned in the material and methods. Is it DNA extraction from the whole tick ?

REPLY: To improve the description of the analyzed ticks, we made several changes:
- Line 108: "All ticks were dissected under a binocular stereo microscope using single-use equipment to avoid contamination.".
- Lines 184-185, the sentence "Four females and eight nymphs were freshly moulted." has been added.

L233 : « *Cox1 gene was successfully amplified from 33 of the 60 organs in which the Babesia sp. YLG 18S rRNA gene was detected (17 salivary glands, 10 ovaries, 6 female endospermatophores, 1 male genitalia and 26 caeca).* » The number of positive caecum are not mentioned before

REPLY: This a good point but the aim of the study was to establish the vectorial competence of *O. maritimus* to transmit *Babesia* sp. YLG. The presence of the parasite in the caeca only attests that the ticks fed on an infected host and not on its ability to transmit the parasite it has ingested. Therefore, we did not insist on the results of detection in caeca (data are available online: https://doi.org/10.57745/XSSMO1). We only mentioned this result in the cox1 sequence analysis to have as many sequences as possible from different haplotypes.

L238-239 : « *on 17 fully characterized sequences* » It is not clear why they are some fully characterized sequence, what it means. From Table 1, we can see that some SNP positions are not characterized. Is it due to sequencing issue. Why ?

REPLY: Sequence quality was sometimes not good enough to get the expected sequence lengths including the different SNPs. We deleted "fully" and added some details in the text: lines 235-238.

L239-240 « *7 "a" variants, 5 "b" variants, 5 "c" variants, 3 "d" variants and 1 "e" variant were counted*. » This does not sum up to 17 sequences. From Table 1, I understand that for some ticks, they are 2 potential variants (that are read in the same sequence ?) . Therefore, you may state that you obtained 21 sequences. At least, there is a need for further explanations with those sequences and variants.

REPLY: We propose to delete this sentence because the frequency of the haplotypes might not be relevant to discuss. We cannot conclude if several identical haplotypes characterized from one tick correspond to different isolates or to the same isolate, and therefore if they should be counted as one or as many. We also added some explanation of this problem in this section (lines 242-244).

L 241-242 : « *but sometimes different combinations of haplotypes were detected within the same tick* ». This is already mentioned line 236-237

REPLY: We modified the paragraph (line 234). We hope this is clearer.

L 309 : « *A. vespertilionis » Yo*u should also write the entire genus name.

REPLY: Line 306: "*A. vespertilionis*" has been replaced by "*Argas vespertilionis*".

L 313 : Malhobo et al 2021 : This reference is missing from the reference list.

REPLY: The reference was not in the right place and has been added line 487.

L 314-315 : « *O. capensis ».* Write the genus name. Ornithodoros I guess (you have a Otobius just before so it may be confusing)

REPLY: The genus name "*Ornithodoros*" has been added line 313.

L 327-328 : « *to the oocyte maturation stage at the time of infection allowing or not parasite penetration into oocytes*. » Would you have any bibliographic reference to support this hypothesis ?

REPLY: We added 2 references to describe oocyte maturation (Denardi et al., 2004) and the role of the vitellogenin receptor in the possible transovarial transmission of *Babesia* (Mitchell et al., 2019) (lines 328, 437, 505).

L 330-331 : « *YLG can be transmitted vertically in its soft tick vector and thus whether ticks can maintain local infection rates, at least from one year to the next*. » Is it really required that there is vertical transmission for ticks to maintain local infection from one year to the next ? I guess that the longevity of soft ticks may allow them to transmit pathogens from one host at year N-1 to another host at year N. I see that this idea is presented in your last paragraph. It may be useful to mention it here.

REPLY: We agree with the reviewer and have clarified the text to read "without the required presence of infected host birds" (lines 330-331).

L 390 : « Maxime Duhaydon ». I guess this is Maxime Duhayon ?

REPLY: Thank you for pointing this typing error.

L 566 : « ***SM3.*** *Number of each organ type of Ornithodoros maritimus collected after dissection and used to test for the presence of Babesia sp YLG (number of ticks in brackets).* » In Mat & Meth, you mentioned the dissection of caecum. Are they dissected in all tested ticks ? If so, could you state it clearly ?

REPLY: In SM2 (previously SM3) we added a column for caeca results, as well as for all other organs: https://doi.org/10.57745/XSSMO1. It is now stated in the Mat & Meth that all ticks were dissected (line 108).

Response to Tahar Kernif

Comments of Reviewer :

The present manuscript reports an interesting study conducted on Ornithodoros maritimus isolated from the Yellow-legged gull in order to estimate the epidemiological action and the propagation of Babesia sp. which is considered as the unique blood parasite species previously detected in gull chicks in the colony.

it is really a very complete study because several parameters concerning the infection of ticks by this endoparasite (Babesia sp.) were studied, including dissection of fresh ticks to isolate different organs and test for the presence of the parasite using molecular assays. The main results raise the interesting possibility of sexual transmission of Babesia sp. from infected males to uninfected females and also the transovarial transmission of the parasite.

The paper is very well written, however certain technical details are needed to make the work more rewarding in terms of data.

Line 100. In the study location section, it would be interesting to add the climatographic conditions of the study area

REPLY: We have now added “Carteau is a small sandy islet within the Gulf of Fos in southern France with a Mediterranean climate, typically characterized by wet autumns and winters, and warm, dry summers.” (lines 96-98).

Line 128-177. the presentation of this part needs to be completely reviewed

· Line 129. In the molecular study section, replace 18S r RNA by 18S r DNA

REPLY: This modification has been made in the entire manuscript.

· Line 131; 134; 146; 158; 171. put all primers in a table not in supplementary Material.

REPLY: As requested, we added all the primers in a table (Table 1) (line 175).

· Line 133. I think it is better to detail the composition of the 30 µL PCR reaction mix and nested PCR cycling than to refer to a reference, especially since this is the first part of the molecular detection and characterization of piroplasms.

REPLY: As recommended, we described the nested PCR conditions (lines 127-134)

· Line 140. Controls !!! a title to change or remove

REPLY: We removed the title “5. Controls”

· Line 141-144. the purpose of this alignment is unclear

REPLY: This allows the readers to check easily for primer specificity. Thus we added this text: “Regions specific to each organism were selected to design the control primers as described in Supplementary Material 1.” (lines 141-142)

· Line 142. The access number “OP542291” doesn't match with O. maritimus! https://www.ncbi.nlm.nih.gov/nuccore/OP542291

REPLY: Sorry and thank you for noticing this error. The access number has been corrected: OP542591.

· Line 145. why an 18S rRNA gene for the molecular identification of O. maritimus not 16S or 12S mitochondrial gens?

REPLY: There is little sequence data for 16S and 12S mitochondrial genes of *O. maritimus* available in GenBank. We obtained a 1763 bp 18S rDNA sequence of *O. maritimus* in our lab, allowing us to design specific primers to check for the presence of tick DNA in each sample and confirm the success of our DNA extractions. For this reason, we preferred using the 18S rDNA fragment as a control.

Line 181. In Ticks collections results I don’t understand the meaning of (Bonsergent, 2023)

REPLY: Thank you for noticing this copy/paste error which has been deleted.

Line 193. it is always necessary to support the results with statistical tests to estimate the significance of the data obtained. even more, do not forget to cite figures and tables in the text

REPLY: We did not initially perform statistical tests as our aim was to qualitatively evaluate the transmission of *Babesia* sp. YLG by different tick stages of *O. maritimus*. Ticks were collected in several consecutive years to check the persistence of infection in the Carteau islet. We never intended to compare results from one year to another or from one tick stage to another. Indeed, in 2020 with covid pandemic, ticks were not analyzed as quickly as the other years which might have influenced the data. However, we have now added in these tests, highlighting that precaution is necessary (see lines 198-201).

Line 198. proceed with the same way 'percentage and ratio with positive ticks coming from

REPLY: We are not sure to understand the reviewer’s comment. We have changed the sentence to homogenize the data presentation to: “All tick stages were found infected, with adults tending to show higher prevalence than nymphs (p-value = 0.07875): 31.8% of females (34/107), 26.7% of males (4/15) and 9.1% of nymphs (2/22).” (lines 201-203). We hope this corresponds to the reviewer expectation.

Line 232. The study of variability of the cox1 gene lacks precision concerning the haplotyping method used (manual, or with bioinformatics tools such as DnaSP)

REPLY: The haplotyping method of *cox1* gene was manual. We have added this detail to the text (line 174).

Table 1. What does the ? mean? as well as the Y's and the R's

REPLY: We added the meaning of ?, Y and R in the legend of the table (Table 2).

The best way to present haplotyping result would be the phylogenetic construction which allows to visualize the clusters relating to each haplotype

REPLY: The phylogenetic analysis of the *cox1* haplotypes has already been published in a previous paper. As no new haplotypes were detected here, we don't think that this analysis would provide any new information. Our aim here was to highlight that the same haplotypes are found in both ticks and YLG hosts.

It would be also interesting to specify Haplotype (gene) diversity (Hd), Nucleotide diversity (Pi) and Estimate the Evolutionary Divergence between Sequences

REPLY: This is a very interesting comment. This kind of study would be particularly informative when more *cox1* sequences from related parasites are available from different geographical origins. We hope to address this variation in more detail in future work.