New insights into the role of the microbiota in *Ornithodoros moubata*: Influence of endosymbionts on the reproductive fitness of the tick

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ABSTRACT

Over the past ten years, many studies demonstrated the crucial role of the tick microbiome in tick biology. The soft tick *Ornithodoros moubata* is a hematophagous ectoparasite of *Suidae* particularly known to transmit the *African swine fever virus*. Its bacterial microbiota is characterized by a high prevalence of *Francisella*-like and *Rickettsia* endosymbionts. The present study aims to better understand the potential influence of the microbiota on the reproductive fitness of *O. moubata*. A total of 132 adult female ticks were treated using gentamycin or rifampicin added to the blood meal. Half of the ticks also received a supplementation with B vitamins to address the nutritional role of endosymbionts. Over two periods of 50 days, several traits related to the reproductive fitness were monitored to investigate the importance of *Francisella* and *Rickettsia* for those traits. It appeared that most of the considered reproductive parameters were not affected. However, antibiotic treatments induced an increase in the tick survival indicating a potential fitness cost of harboring endosymbionts for ticks during the reproduction period. Similarly, 366 first stage nymphs of *Ornithodoros moubata* were exposed to the same treatments for molecular quantification of both endosymbionts. Results from qPCR suggested that treatments produced a bacteriostatic effect on endosymbionts without fully eliminating *Francisella* or *Rickettsia*.

Keywords: Soft tick, *Ornithodoros*, microbiota, antibiotic treatment, *Francisella*-like endosymbiont, *Rickettsia*

Introduction

While ticks are among the most important vectors of pathogens affecting humans and animals worldwide, it is now well established that tick-borne pathogens (TBPs) coexist with many other microorganisms in ticks (e.g., Bonnet *et al*. 2017; Estrada-Peña *et al*. 2018). These other tick-borne microbes (microbiota) are likely to confer multiple detrimental, neutral, or beneficial effects to their tick hosts (Duron *et al*. 2018; Duron, Cremaschi, et McCoy 2016). In addition, many studies identified multiple interactions between some members of the tick microbiota and TBPs (e.g., Lejal *et al*. 2021; Aivelo, Norberg, et Tschirren 2019). For instance, these tick microbial communities can play a role in the transmission or multiplication of TBPs (Vayssier-Taussat *et al*. 2014; Bonnet *et al*. 2017; Budachetri *et al*. 2018; Bonnet et Pollet 2021) which question about the future possibility to manipulate tick microbiomes and use them as a new tool in tick and tick-borne disease control.

If the precise role of all tick-borne microbial species remains unclear, they can be broadly classified as environmental or endosymbiotic. Those later intracellular bacteria are further categorized into primary and secondary endosymbionts according to their importance for the host (Jiménez-Cortés *et al*. 2018), although intermediate forms are acknowledged. Primary symbionts are heritable and obligate symbionts and represent crucial members of the microbiota for the survival or the development of the arthropod (Bonnet *et al*. 2017). In contrast, secondary endosymbionts are not required for host survival and are more sporadically associated with host individuals. Accordingly, four main bacterial genera are currently well identified as tick primary endosymbionts: *Coxiella*-like endosymbionts (CLE), *Candidatus Midichloria mitochondrii*, *Francisella*-like endosymbionts (FLE), and, less often, *Rickettsia*. All of these endosymbionts were identified to carry a copy of genes involved in essential nutrients synthesis, including B vitamins which are not naturally brought by the strictly hematophagous alimentation. Depending on the tick species, one or two of these genera will be found predominantly and will assume the nutritional function (Duron et Gottlieb 2020; Bonnet et Pollet 2021). For example, this role is assumed by *Coxiella*-like endosymbionts (CLE) in *Amblyomma americanum* whereas it is assumed by *Rickettsia* in *Ixodes pacificus* (Rio, Attardo, et Weiss 2016). Over time, several evolutionary events of loss and/or new endosymbiosis are suspected, leading to the diversity of endosymbionts currently found (Duron *et al*. 2017; Duron et Gottlieb 2020).

However, the role of primary endosymbionts is not limited to nutrient supply. In ticks, they are also known to have an influence on fitness, reproduction (Guizzo *et al*. 2017; Li, Zhang, et Zhu 2018; Zhang *et al*. 2017; Zhong, Jasinskas, et Barbour 2007; Kurlovs *et al*. 2014), immunity, and vector competence (Narasimhan *et al*. 2014; Bonnet et Pollet 2021; Gall *et al*. 2016). Besides primary endosymbionts, many other members of the microbiota revealed by high-throughput sequencing are still poorly known and their effects on tick biology are not elucidated yet.

The life cycle of soft ticks (several nymphal stages, short blood feedings, adult females feeding and laying eggs several time before dying) makes these ticks an ideal model for studying the role of the tick microbiota on tick biology and physiology. *Ornithodoros moubata* is a soft tick widely distributed in Southern and Eastern Africa (Vial 2009; Leeson 1952). Its main hosts include wild and domestic members of the *Suidae* family: warthog, bushpig and domestic pig (Peirce 1974). This tick species is mainly known as a reservoir host for the *African swine fever virus* (ASFV) and is consequently an important concern for animal health issues (Sánchez-Vizcaíno *et al*. 2015).

High-throughput 16S rDNA sequencing in *O. moubata* showed that the most abundant bacterial species belonged to the genus *Francisella* and *Rickettsia* (Duron *et al*. 2018). Both bacteria are recognized as maternally-inherited endosymbionts in ticks (Duron *et al*. 2017) Furthermore, *Francisella*-like endosymbionts (FLE) have been shown to play a key role in the production of B vitamins, including biotin, folic acid and riboflavin (Duron *et al*. 2018).

Apart from their role in tick nutrition, the influence of *Francisella* and *Rickettsia* in the physiology and life history traits of soft ticks remains poorly known. In comparison, past studies suggest a role of CLE and *Rickettsia* for the reproductive fitness of female hard ticks belonging to the genera *Ixodes* (Kurlovs *et al*. 2014), *Rhipicephalus* (Li, Zhang, et Zhu 2018), *Haemaphysalis* (Zhang *et al*. 2017), and *Amblyomma* (Zhong, Jasinskas, et Barbour 2007) which for the reproductive fitness (number of eggs laid, mass of eggs, duration of oviposition and hatching rate) was negatively impacted by the elimination of parts of the microbiota.

The present study aimed to better characterize the role of the microbiota in reproductive fitness in soft ticks. Accordingly, our first objective was to eliminate the main bacterial species of the microbiota of

Ornithodoros moubata with antibiotic treatments. The second goal was to evaluate the consequences of such a disruption on the reproductive fitness of ticks, using B vitamin supplementation to identify which part of the observed changes was directly attributable to an action of the endosymbionts on the reproduction and which part was a consequence of their nutritional role.

Material and methods

Ticks

All ticks are issued from a colony maintained at the CIRAD laboratory (Montpellier, France, member of the Vectopole Sud network) since 2008. This colony originated from the Neuchâtel strain initially collected in Tanzania and maintained at the University of Neuchâtel (Switzerland).

The female ticks used for the experiments had never been mated before and did not receive any blood meal since molting from the last nymphal stage to the adult stage.

Adult ticks, nymphs, larvae, and eggs were kept in stable conditions in one incubator, at 25°C, 85% relative humidity, and 0% luminosity.

Feeding and antibiotic treatment

Adult stages were engorged with a first blood meal at the beginning of the experiment and a second one 52 days later. Blood meals were performed using an artificial feeding system with fresh cow blood, collected 1-3 hours before the experiment in 10mL heparinized tubes. All the blood used for this experiment was issued from the same cow. Blood meals lasted three hours in complete darkness while blood temperature was maintained at 41°C and stirred at 250 rpm.

Antibiotics and B vitamins were added in the blood for each group following the protocol of Duron *et al.* (Duron *et al*. 2018) except that dilutions were performed in Milli-Q water instead of methanol due to the potential toxicity of methanol for arthropods (Kaviraj, Bhunia, et Saha 2004). Concentrations were chosen according to recommendations for sensitive bacterial species and previous studies in the literature: gentamycin 10 $\mu g.mL^{-1}$, rifampicin 10 $\mu g.mL^{-1}$, biotin 1 $\mu g.mL^{-1}$, folic acid 30 $\mu g.mL^{-1}$, riboflavin 20 $\mu g.mL^{-1}$ (Duron *et al*. 2018; Zhong, Jasinskas, et Barbour 2007; Narasimhan *et al*. 2014). Antibiotics and B vitamins were replaced by the same volumes of Milli-Q water for negative controls.

Each tick was weighted before and after the blood meal. To ensure efficient blood meal, ticks were fed in group: four to seven females were fed together with 3 males in one well containing 9 mL of blood. Therefore, we had to mark ticks individually with different colors using Posca felt pens. If ticks gained less than 50 mg at the end of the first blood meal, the blood feeding was considered as a failure and ticks were fed again during a second, and, if needed, a third blood meal. If after three attempts some ticks were still not fed, they were excluded from the study.

Independently from the feeding of adults, nymphal stages were engorged by groups of thirty individuals. Treatment groups were the same as the one used for adult ticks (control, rifampicin, gentamycin, with or without B vitamins) with a total of 60 nymphs for each condition. A total of 20 individuals were sampled for molecular analyses at day 0 (before blood meal), and 10 individuals at day 1, day 3, day 5, day 10, day 15 and day 30 after blood meal (**Figure 1**). Nymphs which died before DNA extraction were excluded. For sample size and group size, see supplementary data (**Table S1**).

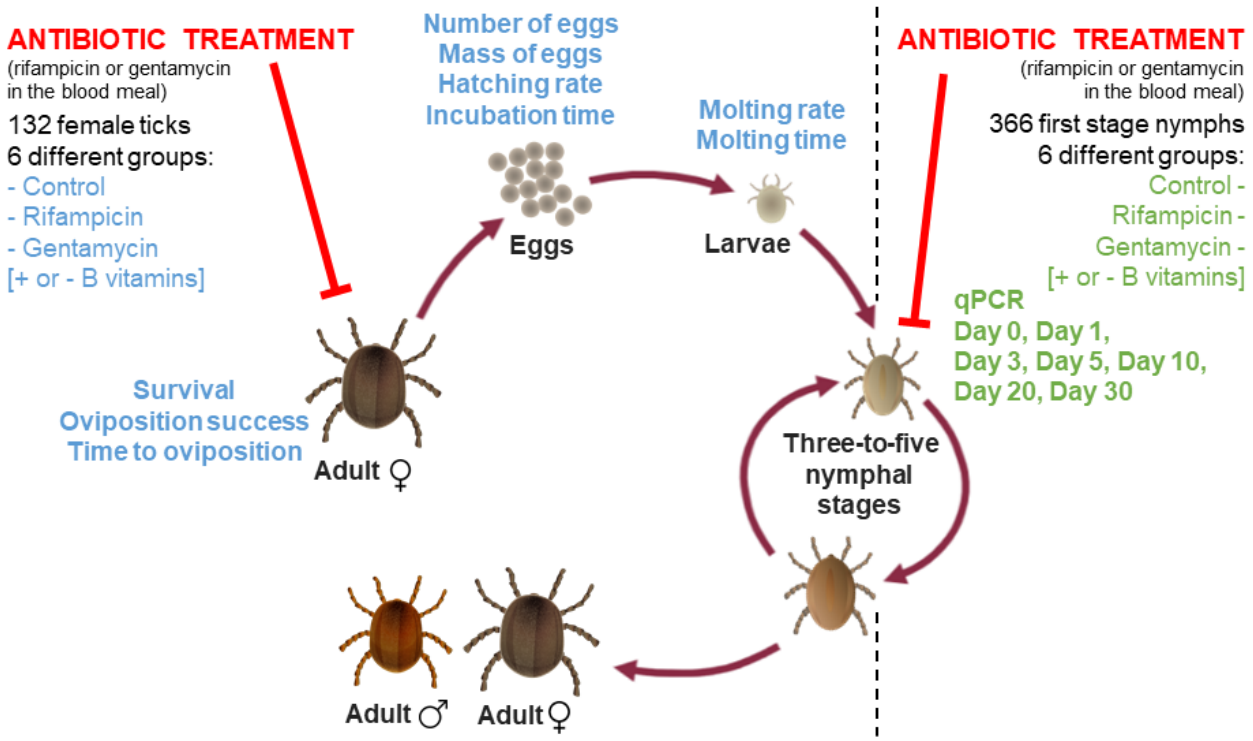

Figure 1: Antibiotic treatments performed, followed by the evaluation of traits of interest at different stages of the parasitic cycle of *Ornithodoros moubata*. The traits monitored are indicated near the parasitic stage concerned. In blue, experiment conducted on adult ticks to monitor reproductive parameters. In green, experiment conducted on first stage nymphs to monitor bacterial loads after antibiotic treatment. Tick pictures: Françoise Chirara © Cirad-ASTRE.

Reproduction monitoring

After blood meal, females were kept in the same tubes as males for seven days to allow mating, then each female was isolated in an individual plastic tube. The monitoring of the tick reproduction was blinded regarding applied treatments. A total of 132 females were included in the study and monitored every day during two successive 50-days periods after blood meal. For each tick, the laying date, the number of eggs laid and the egg mass were recorded. The number of eggs laid was standardized with the weight of the female tick after blood meal. For each egg, the date of hatching and of molting into first nymphal stage were recorded (**Figure 1**). At the end of the experiment, the remaining females were stored at -80°C for molecular analyses.

DNA extraction

Nymphs and adults were washed 30 seconds in 1% bleach solution and then rinsed for 1 minute in three successive baths of Milli-Q water to eliminate cuticular bacteria (Binetruy *et al*. 2019). Ticks were then crushed individually using a Qiagen TissueLyser instrument with steel and plastic beads in 300 µL of DMEM medium complemented at 10% with calf serum. DNA was extracted from 75 µL of each crushed tick homogenate, using the genomic DNA extraction kit NucleoSpin® Tissue (Macherey-Nagel, Hoerdt, Germany), and following the standard protocol for human or animal tissue and cultured cells. DNA extracts were finally eluted in 50 µl of elution buffer and stored at − 20 °C until further use.

Quantitative PCR

Quantitative PCRs were launched using Roche Lightcycler® 96 instruments (Roche Applied Sciences, Beijing, China) using the taqMan method.

Extracted DNA (2 µL) was added to the amplification mix (18 µL: Mix 10 µL, H2O 6.6 µL, forward Primer Actin 0.6 µM, reverse Primer Actin 0.6 µM, Probe Actin 0.2 µM, forward Primer target gene 0.6 µM, reverse Primer target gene 0.6 µM, Probe target gene 0.2 µM). Primers used to amplify *Francisella* targeted the *RpoB* gene coding for the B subunit of RNA polymerase. Primer sequences were Forward: 5'-GTGGTGTACCTATTGCTACG-3', Reverse: 5'-TGATGGTCGTACTGGTAAGC-3' and Probe: 5'-ATAGGTGGCTTTGCAACTAA-3'. Primers used to amplify *Rickettsia* targeted the *GltA* gene coding for the citrate synthase enzyme. Primer sequences were Forward: 5'-AATGATCCGTTTAGGTTGATG-3', Reverse: 5'-ACCCACTCCTGCAAATAG-3' and Probe: 5'-GTGCCGCAGTACTTAAAGA-3'. The actin gene from *Ornithodoros moubata* was also amplified as a control for the assessment of DNA extraction success and to standardize the results with the tick size. Primer sequences were Forward: 5'-CCGGTATTGCCGACCGTATGC-3', Reverse: 5'-CTCCCTGTCCACCTTCCAGC-3' and Probe: 5'-CGAGAGGAAGTACTCCGTCTGG-3' (Pereira De Oliveira, Vial, et Le Potier 2022; Duron *et al*. 2018).

Positive and negative controls were included, using 2 µL of DNA of tick previously tested positive for *FLE* and *Rickettsia* (Duron *et al*. 2018) and 2 µL of Milli-Q water respectively. The PCR cycling program was 1 min at 95°C, then 45 cycles of 1 min at 95°C, 30 sec at 56°C. Analyses were performed using both FAM and HEX fluorescence indicators. Samples with a cycle threshold (Ct) beyond 38 were considered as negative. Cts were converted into concentrations from the standard curves performed with dilutions of a DNA plasmid containing the targeted sequences. Results are presented as ratio of the concentration of the gene of interest (GltA or RpoB) divided by the concentration of the actin gene to standardize for the tick size.

Statistical analysis

Statistical analyses were performed using R version 4.2.3 (2023-03-15) to test the effect of antibiotic and vitamin treatments on DNA concentrations and traits associated with reproductive fitness (R Core Team 2023). For the analyses of qPCR results, ratios of DNA concentrations were log-transformed and generalized least-squares (GLS) models were used with the function **gls** to allow different variances for each level of the explanatory variables (Zuur *et al*. 2009). The best model including optimal variance structure and fixed effects (namely, antibiotic and vitamin treatments and their interaction) was determined by AIC comparisons. In nymphs, due to material constraints, two different experimenters performed the qPCR at two different moments. This was added as a fixed effect in all the models and had a clear effect on measured ratios (GltA: $p=1.602e^{-05}$, F-value=19.17 and, RpoB: $p=1.144e^{-65}$, F-value=472.7). DNA concentrations were also log-transformed and analyzed using **glm** with a gamma distribution when ratios were not relevant for the analyzes. To analyze reproduction parameters, we used generalized linear models with the function **lm**, **glm** or generalized mixed model with **glmer** (R package **Lme4** (Bates *et al*. 2015)) according to the distribution of the data and the absence (**glm**) or presence (**glmer**) of a random effect. The random effect accounted for the lack of independence of data points (since measured individuals may be offspring of the same mother), it was added for analyses targeting the offspring of tick females. Depending on the variable, normal, Poisson or binomial distributions were used (see **Table 1**). Overdispersion and underdispersion of the data were estimated using a quasi-Poisson or quasi-binomial distribution. Statistical comparisons were performed using either F-test, chi-squared test or Kruskal-Wallis Test and deviances and p-values were reported. Tick weight and feeding success after bloodmeal was analyzed and no effect of the antibiotic group (first 50 days: p=0.9325 and p=0.5054; second 50 days: p=0.9050 and p=0.5343) nor vitamin group (first 50 days: p=0.5556 and p=0.1553; second 50 days: p=0.9835 and p=0.0892) was reported. When the mean was presented for a trait, standard deviation (SD) was indicated in brackets. In addition, data from the two 50-days periods were statistically compared and analyzed using the models described above. Interactions with antibiotic treatments and B vitamin supplementation were taken into account in the analysis.

Ethics

The use of cows which provided blood for tick engorgement complied with the ethical standards of European regulations (directive from the European Council from November 24th, 1986 (86/609 / EEC)) and French legislation (ethic committee CEEA-LR-36 available on the APAFIS platform) on the care and use of animals. More precisely, blood sampling has been recorded in 2021 under the agreement number APAFIS#27628-2020101010475151 for the CIRAD-Montpellier rearing facility G 34-172-15.

Results

Molecular results

A total of 366 first stage nymphs were tested for *Rickettsia* (target gene *GltA*) and *Francisella*-like endosymbiont (target gene *RpoB*). Out of 366 samples, 98.6% were tested positive for *Rickettsia* (359/364) while two samples were excluded due to the abnormal qPCR curves. Two nymphs were tested negative in the control group without vitamins (at day 3 and day 5) and three others in the gentamycin group (day 30 with and without vitamins and day 10 without vitamins). The concentration of *GltA* DNA increased over time after blood meal in the control (p=0.003528, SD=22.50) and gentamycin (p=4.74e^{-11}, SD=59.70) groups but not in the rifampicin group (p=0.2616, SD=2.821). Similar increase also appeared for the actin gene in all groups (p<2.2e^{-16}, SD=115.5). After standardization, the ratio of DNA concentration remained significantly lower in the rifampicin group than in the two other groups (p=1.624e^{-92}, F-value=429.0), independently from vitamin supplementation (**Figure 2A and 2B**).

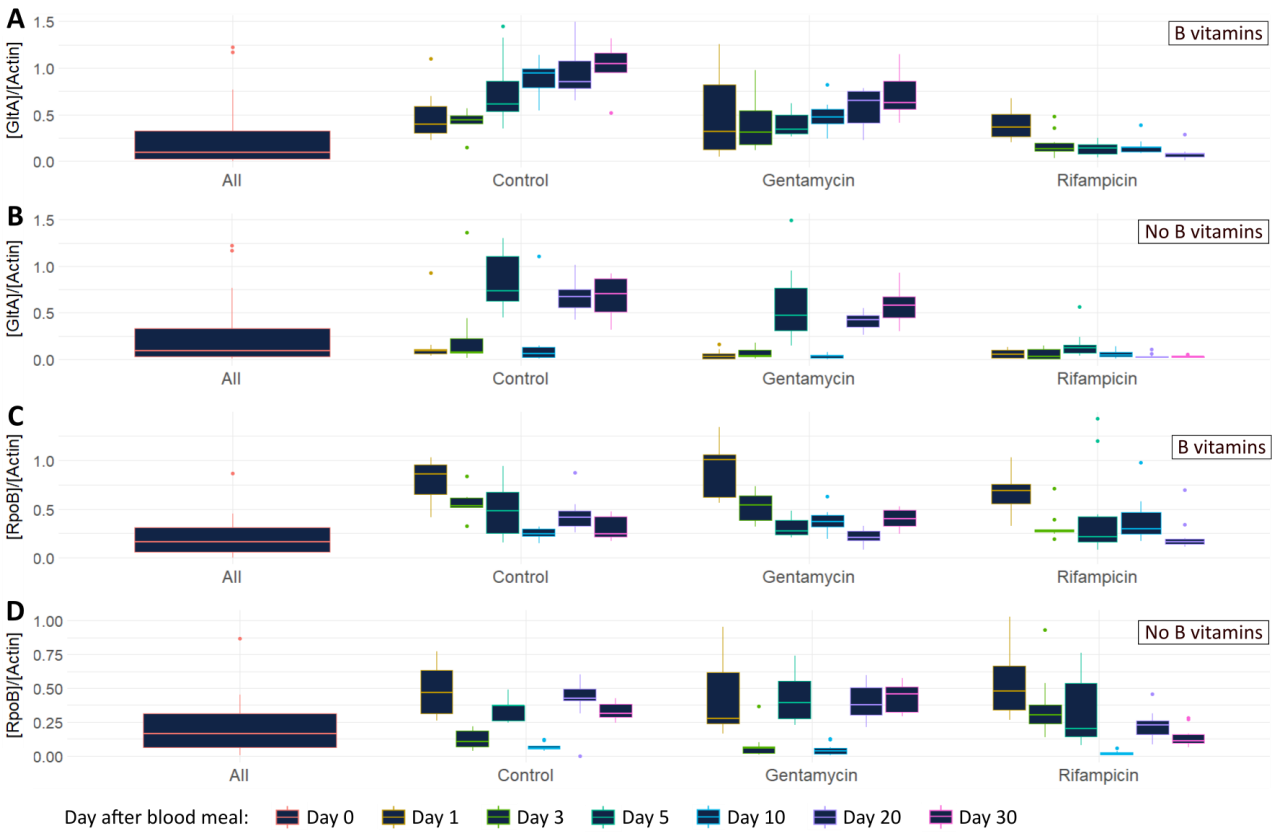

Figure 2: Boxplots of the ratios of DNA concentration obtained by qPCR for *Rickettsia* and *Francisella*-like endosymbiont (FLE) after antibiotic treatment of individual first stage nymphs. (A) Ratio of the concentration of *GltA* DNA ([GltA]), indicator of the presence of *Rickettsia* over the concentration of *O. moubata* actin DNA [Actin]. Nymphs supplemented with B vitamins. **(B)** Ratio of [GltA] over [Actin]. Nymphs not supplemented with B vitamins. **(C)** Ratio of the concentration of *RpoB* DNA ([RpoB]), indicator of the presence of FLE over [Actin]. Nymphs supplemented with B vitamins **(D)** Ratio of [RpoB] over [Actin]. Nymphs not supplemented with B vitamins.

Regarding *Francisella*-like endosymbiont, 98.1% of the qPCR were positive (359/366) with one negative samples at day 0, two in the rifampicin group (day 1 no vitamin, and day 10 with vitamin) and four in the control group (day 5 and 10 with vitamin, and day 20 and 30 no vitamin). As for *Rickettsia*, there was an increase in the quantity of *RpoB* DNA detected over time in the gentamycin group (p=0.003095, SD=23.84) but not in the control (p=0.08688, SD=3.190) and rifampicin (p=0.1337, SD=5.103) groups. After standardization,

the ratio of DNA concentration remained significantly different between the three treatment groups ($p=1.709e^{-12}$, F-value=29.42) (**Figure 2C and 2D**).

At the end of the reproduction monitoring, a total of 59 females were still alive and were collected for quantification of *Rickettsia* and *Francisella*-like endosymbionts. Forty-eight females were tested positive for *Rickettsia* (14/14 control, 2/6 gentamycin without vitamin, 11/12 gentamycin with vitamin, 10/14 rifampicin without vitamin, and 11/13 rifampicin with vitamin), all females were tested positive for *Francisella*-like endosymbiont, and five adults were excluded due to abnormal qPCR curves. Comparing the ratio of concentration of symbionts, no significant differences were observed between the groups concerning vitamin supplementation ($p>0.05$). However, focusing on the different treatment groups, it appeared that ticks treated with rifampicin or gentamycin hosted lower concentrations of *Rickettsia* ($p=1.805e^{-06}$, F-value=19.11) and *Francisella*-like endosymbiont ($p=1.123e^{-09}$, F-value= 31.72) than ticks from the control group. This effect was stronger in the rifampicin group than in the gentamycin group (**Figure 3A and 3B**).

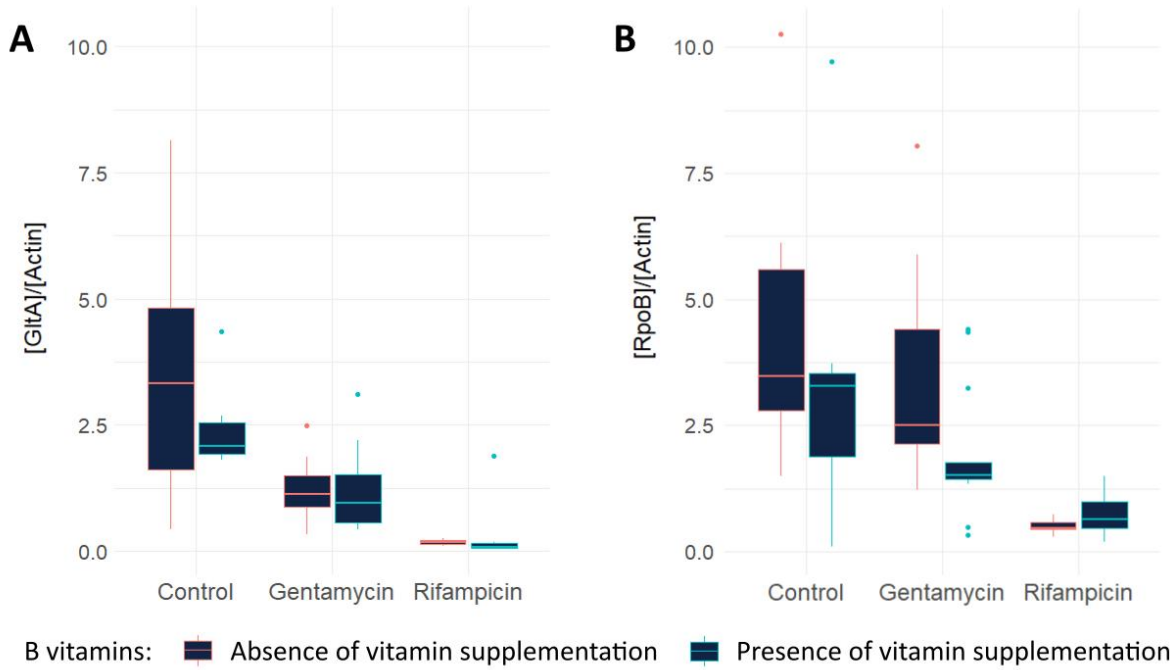

Figure 3: Boxplots of the DNA concentration obtained by qPCR for *Rickettsia* and *Francisella*-like endosymbiont (FLE) after antibiotic treatment of female ticks. (A) Ratio of the concentration of *GltA* DNA ([GltA]), indicator of the presence of *Rickettsia* over the concentration of *O. moubata* actin DNA [Actin]. **(B)** Ratio of the concentration of *RpoB* DNA ([RpoB]), indicator of the presence of FLE over [Actin].

Monitoring of the reproduction

Adult ticks were separated in three treatment groups with two subgroups in each (half with B vitamin supplementation and half without). Effects of antibiotic treatment, vitamin supplementation and the interaction between the two were evaluated for statistical differences on traits associated with reproductive fitness over two successive periods of 50 days (**Table 1 and 2**).

Table 1: Statistical analyses performed on traits associated with reproductive fitness measured in female ticks during 50 days after the first antibiotic treatment and vitamin supplementation.

Variable	Family	Effect of the treatment	Effect of the vitamin	Effect of Interaction
Survival over 50 days after blood meal	Binomial	Pr: 0.009687** Dev: 9.274	Pr: 0.3768 Dev: 0.7810	Pr: 0.1867 Dev: 3.356
Laying probability	Binomial	Pr: 0.3499 Dev: 2.100	Pr: 0.1073 Dev: 2.594	Pr: 0.4483 Dev: 1.605
Number of days to first oviposition	Quasi-Poisson[1]	Pr: 0.3741 Dev: 3.548	Pr: 0.05820 Dev: 6.472	Pr: 0.1609 Dev: 6.590
Number of eggs standardized by mother weight[3]	Quasi-Poisson[1]	Pr: 0.4116 Dev: 18.70	Pr: 0.7698 Dev: 0.9018	Pr: 0.9174 Dev: 1.816
Average egg mass	Normal	Pr: 0.3529 Dev: 0.002267	Pr: 0.1437 Dev: 0.004680	Pr: 0.8892 Dev: $2.531e^{-04}$
Egg incubation time	Quasi-Poisson[2]	Pr: 0.6958 Dev: 0.06174	Pr: 0.7652 Dev: 0.007590	Pr: 0.05697 Dev: 0.4878
Egg hatching rate	Binomial	Pr: 0.7476 Dev: 0.5817	Pr: 0.9567 Dev: 0.002947	Pr: 0.5500 Dev: 1.196
Time for molting into first nymphal stage	Quasi-Poisson[2]	Pr: 0.7098 Dev: 0.04697	Pr: 0.3924 Dev: 0.05011	Pr: 0.7444 Dev: 0.04045
Molting rate	Binomial	Pr: 0.8713 Dev: 0.2756	Pr: 0.1844 Dev: 1.762	Pr: 0.7626 Dev: 0.5419

Note: Pr: P-value. Dev: Deviance. Degree of freedom was 2 for the effect of the treatment and the effect of the interaction and 1 for the effect of vitamin. [1] Distribution corrected for overdispersion of the data. [2] Distribution corrected for underdispersion of the data. [3] Variable corrected using an offset of the tick weight. Statistically significant results are highlighted with a grey background. Statistical difference code: 0 '***' 0.001 '**' 0.01 '*' 0.05 ' ' 1

In total, 78.8% (104/132) of female ticks survived throughout the experiment. Survival was significantly different between the three treatment groups but not between the vitamin subgroups (**Figure 4A**, **Table 1**), with the control group presenting a lower survival rate (65.2%) than both the gentamycin (90.9%) and rifampicin (81.0%) groups.

The oviposition probability was not significantly different between treatment groups (**Figure 4B**, **Table 1**). Out of 132 ticks engorged, 74.2% (98/132) started oviposition during the first period of 50 days. Those ticks laid between 2 and 220 eggs, with an average of 109 eggs, over the first 50 days of monitoring (or, after standardization, 0.361 egg per mg of weight of the tick [SD: 0.116]). No differences between any subgroups were found for this trait (**Figure 4D**, **Table 1**). The mean time between blood meal and first day of oviposition was not affected by treatments with a mean of 23.9 days [SD: 6.81] (**Figure 4C**, **Table 1**). Similarly, no differences were detected for the mean egg mass (**Figure 4E**, **Table 1**). On average, an egg weighted 0.538 mg [SD: 0.0463].

No statistical differences were found between the treatments neither for the egg incubation time, nor for the egg hatching rate (**Figure 4F and 4G**, **Table 1**). Mean values for hatching parameters were 11.9 days of incubation [SD: 1.01] and 79.6% of hatching success [SD: 15.1].

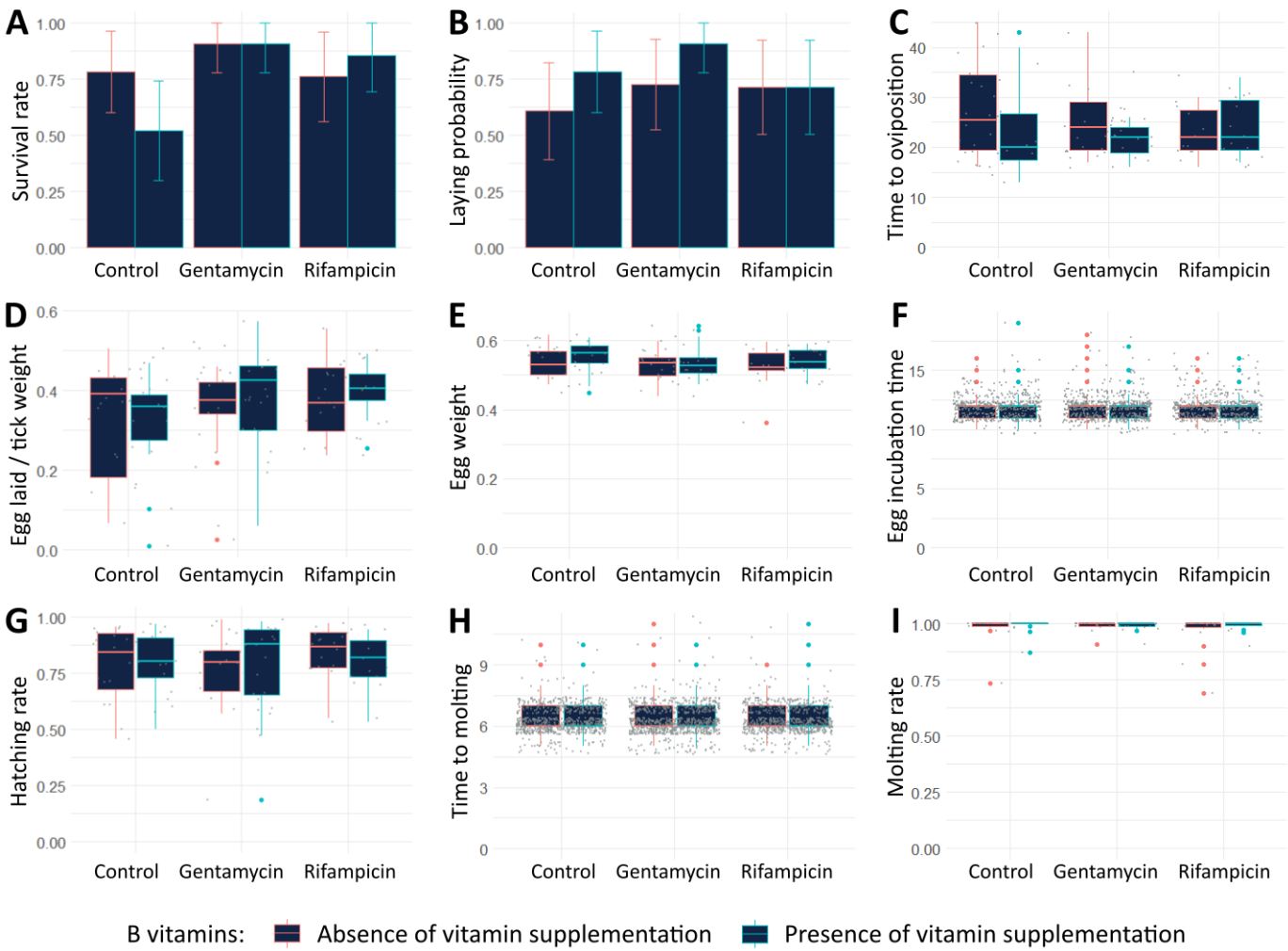

Figure 4: Histograms and boxplots of the traits associated with reproductive fitness measured in female ticks during 50 days after the first antibiotic treatment and vitamin supplementation. (A) Tick survival rate. **(B)** Laying probability: number of ticks laying eggs/ population size. **(C)** Time between blood meal and first day of oviposition (in days). **(D)** Number of eggs laid per tick corrected for the mass of the blood fed female tick (number of eggs per mg of tick weight). **(E)** Mean egg weight (in mg). **(F)** Egg incubation time (in days). **(G)** Egg hatching rate. **(H)** Time between hatching and molting into first nymphal stage (in days) **(I)** Molting success rate. In histograms, statistical significance is indicated: 95% confidence interval.

No differences were registered for the time needed to molt into first nymphal stage (**Figure 4H**, **Table 1**). On average, larvae successfully molted in 6.30 days [SD: 0.657]. Finally, no significant differences were observed for molting rates which reached 98.4% on average [SD: 4.77]. (**Figure 4I**, **Table 1**).

After the first period of 50 days, the surviving ticks were fed a second time with the same feeding protocol. Female ticks were monitored during a new period of 50 days and parameters associated with reproduction were measured (**Table 2**).

Table 2: Statistical analyses performed on traits associated with reproductive fitness measured in female ticks during 50 days after the second antibiotic treatment and vitamin supplementation.

Variable	Family	Effect of the treatment	Effect of the vitamin	Effect of Interaction
Survival over full duration of the experiment (102 days)	Binomial	Pr: 0.006548** Dev: 10.06	Pr: 0.9598 Dev: 0.002503	Pr: 0.06723 Dev: 5.399
Survival over 50 days after the second blood meal	Binomial	Pr: 0.1501 Dev: 3.793	Pr: 0.7106 Dev: 0.1376	Pr: 0.04820* Dev: 6.065
Laying probability	Binomial	Pr: 0.08903 Dev: 5.146	Pr: 0.7287 Dev: 0.1280	Pr: 0.2472 Dev: 2.974
Number of days to first oviposition	Quasi-Poisson[1]	Pr: 0.6820 Dev: 4.207	Pr: 0.1323 Dev: 12.46	Pr: 0.2084 Dev: 17.24
Number of eggs standardized by mother weight[3]	Quasi-Poisson[1]	Pr: 0.2587 Dev: 132.4	Pr: 0.3037 Dev: 51.79	Pr: 0.01714* Dev: 398.1
Average egg mass	Normal	Pr: 0.9865 Dev: 0.0000662	Pr: 0.8609 Dev: 0.0001511	Pr: 0.3596 Dev: 0.005115
Egg incubation time	Quasi-Poisson[2]	Pr: 0.3250 Dev: 0.2497	Pr: 0.6804 Dev: 0.01885	Pr: 0.01565* Dev: 0.9236
Egg hatching rate	Binomial	Pr: 0.7355 Dev: 0.6145	Pr: 0.1896 Dev: 1.721	Pr: 0.4796 Dev: 1.470
Time for molting into first nymphal stage	Quasi-Poisson[2]	Pr: 0.0003831*** Dev: 1.264	Pr: 0.6249 Dev: 0.01920	Pr: 0.9009 Dev: 0.01676
Molting rate	Binomial	Pr: 0.2100 Dev: 3.121	Pr: 0.717 Dev: 0.1314	Pr: 1.000 Dev: $6.72e^{-5}$

Note: Pr: P-value. Dev: Deviance. Degree of freedom was 2 for the effect of the treatment and the effect of the interaction and 1 for the effect of vitamin. [1] Distribution corrected for overdispersion of the data. [2] Distribution corrected for underdispersion of the data. [3] Variable corrected using an offset of the tick weight. Statistically significant results are highlighted with a grey background. Statistical difference code: 0 '***' 0.001 '**' 0.01 '*' 0.05 ' ' 1

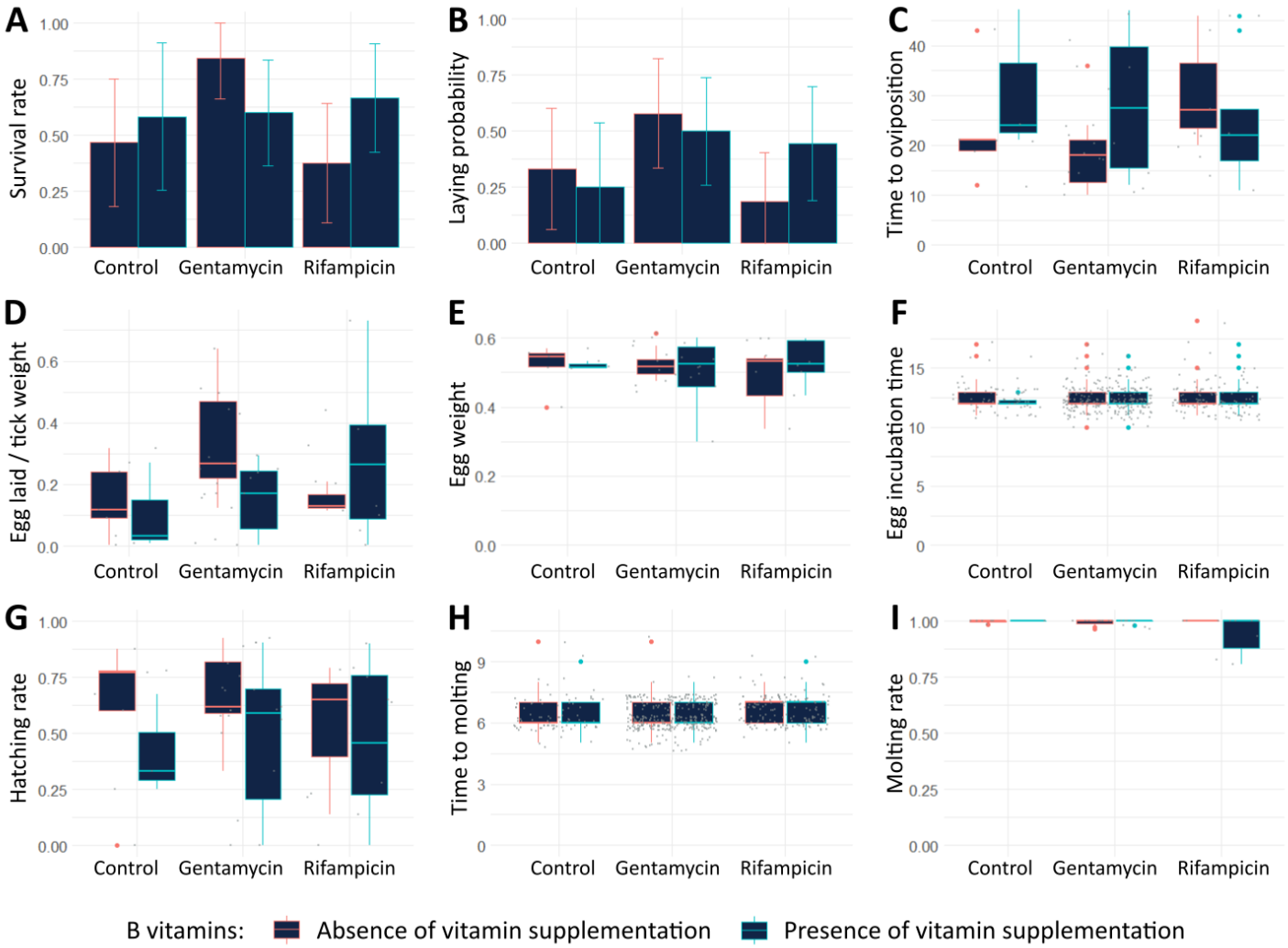

Figure 5: Histograms and boxplots of the traits associated with reproductive fitness measured in female ticks during 50 days after the second antibiotic treatment and vitamin supplementation. (A) Tick survival rate. **(B)** Laying probability: number of ticks laying eggs/ population size. **(C)** Time between blood meal and first day of oviposition (in days). **(D)** Number of eggs laid per tick corrected for the mass of the blood fed female adult tick (number of eggs per mg of tick weight). **(E)** Mean egg weight (in mg). **(F)** Egg incubation time (in days). **(G)** Egg hatching rate. **(H)** Time between hatching and molting into first nymphal stage (in days) **(I)** Molting success rate. In histograms, statistical significance is indicated: 95% confidence interval.

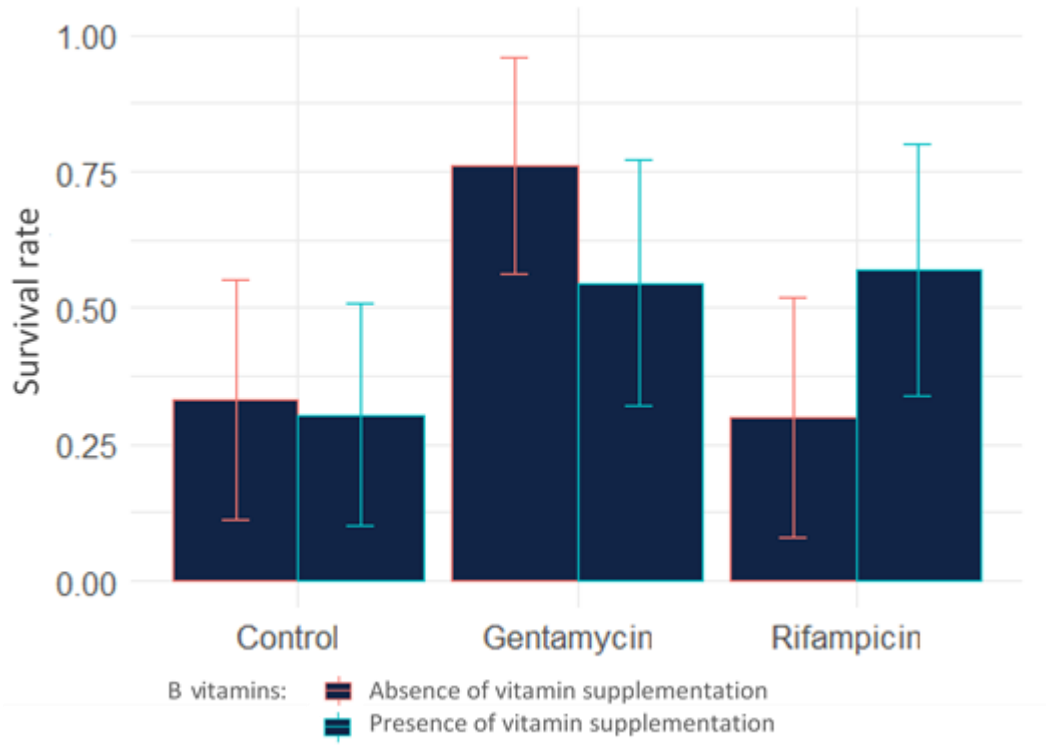

Figure 6: Histogram of the survival rate of *Ornithodoros moubata* ticks after antibiotic treatment and vitamin supplementation over the whole experiment (102 days). Statistical significance is indicated: 95% confidence interval.

Measurements after the second blood meal yielded largely similar results (**Figure 5**, **Table 2, Table S2**). There was no effect of antibiotic and vitamin treatments, except for the time before molting which was increased for larvae of the rifampicin group (6.59 days against 6.38 for the control group and 6.34 for the gentamycin group). No effect was observed regarding vitamin supplementation (**Figure 5H**, **Table 2**).

Considering the whole experiment (two blood meals), a strong effect of the antibiotic treatment group is revealed on survival. The control groups presented some of the lowest survival rates (33.3% and 30.4% respectively in absence and presence of vitamins). The rifampicin group without B vitamins also presented a low survival rate (30.0%), whereas the three other groups presented higher survival rates: 57.1% for rifampicin with vitamin, 54.5% and 76.2% for the gentamycin group with or without vitamins (**Figure 6**, **Table 2)**.

Following the ticks over two consecutive blood meals brings information on how the tick reproduction parameters are affected over time in *Ornithodoros moubata* (**Table 3, Table S2**). Almost all traits associated with reproductive fitness were negatively affected during the second 50-days period after blood meal: survival rates, laying probability, number of eggs laid, egg mass and hatching rate decreased whereas egg incubation time and molting time increased. The number of days to oviposition and the molting rate did not change after the second blood meal. These observations are mostly independent from the treatment received during blood meals.

Table 3: Comparison of the reproductive indicators measured in adult female ticks between the two 50 days periods after blood meal with antibiotic treatment (gentamycin, rifampicin, or negative control) and vitamin supplementation (presence of vitamins and absence of vitamins).

Variable	Family	Effect of Aging	Effect of Interaction with Antibiotic	Effect of Interaction with Vitamin
Survival over 50 days after blood meal	Binomial	Pr: 0.001892** Dev: 9.652	Pr: 0.4465 Dev: 1.613	Pr: 0.4004 Dev: 0.7072
Laying probability	Binomial	Pr: $1.208e^{-07}$ *** Dev: 28.01	Pr: 0.8867 Dev: 0.2404	Pr: 0.3864 Dev: 0.7502
Number of days to first oviposition	Quasi-Poisson[1]	Pr: 0.5998 Dev: 0.8048	Pr: 0.8483 Dev: 0.9622	Pr: 0.008667** Dev: 20.15
Number of eggs standardized by mother weight[3]	Quasi-Poisson[1]	Pr: 0.0004472*** Dev: 327.6	Pr: 0.1917 Dev: 87.80	Pr: 0.1817 Dev: 47.39
Average egg mass	Normal	Pr: 0.03459* Dev: 0.01311	Pr: 0.9348 Dev: 0.001939	Pr: 0.6345 Dev: 0.006528
Egg incubation time	Quasi-Poisson[2]	Pr: $2e^{-16}$ *** Dev: 7.352	Pr: 0.5287 Dev: 0.1153	Pr: 0.5771 Dev: 0.02813
Egg hatching rate	Binomial	Pr: $<2e^{-16}$ *** Dev: 187.2	Pr: 0.0229* Dev: 7.553	Pr: 0.3265 Dev: 0.9629
Time for molting into first nymphal stage	Quasi-Poisson[2]	Pr: 0.009203** Dev: 0.5013	Pr: 0.0002558*** Dev: 1.223	Pr: 0.3808 Dev: 0.05678
Molting rate	Binomial	Pr: 0.9751 Dev: 0.09712	Pr: 0.2268 Dev: 2.697	Pr: 0.3196 Dev: 0.9905

Note: Pr: P-value. Dev: Deviance. Degree of freedom was 1 for the effect of aging and 2 for the effect of interactions. [1] Distribution corrected for overdispersion of the data. [2] Distribution corrected for underdispersion of the data. [3] Variable corrected using an offset of the tick weight. Statistically significant results are highlighted with a grey background. Statistical difference code: 0 '***' 0.001 '**' 0.01 '*' 0.05 ' ' 1

Discussion

Many experts on ticks, tick-microbe interactions and tick-borne diseases now consider that the tick microbiota manipulation might become a potenital new tool to fight against tick and tick-borne pathogens in the future (e.g., Bonnet *et al*. 2017; Bonnet et Pollet 2021). Before considering such an eventuality, it is essential to identify members of the tick microbiota and their role on both tick biology and physiology. It is also crucial to experimentally estimate consequences of the tick microbiota modifications on ticks. In this study, we focused on the microbiota of the tick *Ornithodoros moubata. O. moubata* is known to host two main bacterial endosymbionts, *Francisella* (namely *Francisella*-like endosymbionts (FLE)) and *Rickettsia*, with FLE being the one harboring the nutritional function (Duron *et al*. 2018). Here, using antibiotic treatments, we tried to eliminate members of *O. moubata* microbiota, particularly these two obligate endosymbionts, to evaluate the consequences of this dysbiosis on several parameters of the tick biology.

Impacts of antibiotics in an experimental design

Based on previous studies, we decided to use a protocol with an antibiotic treatment in the blood meal instead of using antibiotic injections or hosts treated with antibiotics (Duron *et al*. 2018; Guizzo *et al*. 2017; Zhang *et al*. 2017; Ninio *et al*. 2015). As a lab species since decades, *O. moubata* presents excellent results with artificial feeding and this method was chosen for easier use and more accurate control of antibiotic concentrations. The protocol used by Duron *et al.* (Duron *et al*. 2018) was chosen as a model and adapted for our study. Another option which was ruled out here, was the use of microinjections to treat the ticks (Guizzo *et al*. 2017; Li, Zhang, et Zhu 2018; Zhang *et al*. 2017; Zhong, Jasinskas, et Barbour 2007; Kurlovs *et al*. 2014; Ninio *et al*. 2015). While this method is extremely efficient to ensure that the right amount of antibiotics is administered, it remains traumatic for the tick and is not adapted for soft tick species (S. Bonnet et Liu 2012).

The choice of the antibiotic molecules was based on the target symbionts. Duron *et al.* recently sequenced the lineage of *O. moubata* from the Cirad laboratory (Duron *et al*. 2018). Based on this study, we decided to use rifampicin which can eliminate both Rickettsia and FLE, and gentamycin, for which most *Rickettsia* strains are resistant whereas *Francisella* species are sensitive. Other molecules such as kanamycin and tetracycline were ruled out due to their potential toxicity for arthropods (Li, Zhang, et Zhu 2018; Coon, Brown, et Strand 2016). Finally, the vitamin supplementation was chosen based on the identification of essential B vitamins (riboflavin, folic acid and biotin) (Duron *et al*. 2018).

Impacts of antibiotics on the tick microbiota

Performing our study and based on previous studies, several observations were expected. First, the elimination of *Francisella* alone under gentamycin treatment would allow to evaluate its role independently from *Rickettsia* (for which insights would be given using rifampicin treatment). However, the role of the rest of the microbiota would still need to be considered and evaluated. Another important methodological aspect is the use of vitamin supplementation. Indeed, if an effect observed on reproductive parameters under antibiotic treatment is suppressed when supplementing with vitamins, it suggests that this change was linked to the nutritional role of the microbiota. On the opposite, if an effect is not rescued by supplementing in vitamins, it is more likely to be the consequence of a direct role of the symbiont on the reproduction.

The experiment performed on first stage nymphs brought interesting results on the effect of the blood meal on the bacterial population size. Indeed, for both *Francisella*-like endosymbionts and *Rickettsia*, a significant increase in the amount of bacterial DNA was observed during 30 days after blood meal. It also appeared that the amount of actin DNA increased over the same period of time, suggesting a strong metabolic activity with multiple cellular divisions. This is most probably linked to the molting process which takes 10.5 days [SD: 1.06] under those conditions (Jourdan-Pineau, unpublished data). As intracellular bacteria, it is possible that *Francisella* and *Rickettsia* took advantage of this metabolic increase for their own growth as observed in other species (Sassera *et al*. 2008). Those endosymbionts might be simply more active during this period of intense metabolic activity which would be in agreement with the alterations of the molting process observed after antibiotic treatments (Duron *et al*. 2018).

Focusing on ticks treated with antibiotics, first stage nymphs showed contrasted responses according to the symbiont considered. Over the 30 days after treatment, no effect of the gentamycin was detected on *Rickettsia* populations. This result was expected for the gentamycin treatment group as *Rickettsia* are known to be resistant to this antibiotic (Vanrompay *et al*. 2018). Conversely, the rifampicin treatment significantly lowered *Rickettsia* population size over time compared to the control group. Focusing on the bacterial concentration instead of the ratios, it appeared that the concentration of *Rickettsia* slightly decreased or stayed at the same level as it was at day 0. This might suggest a strong bacteriostatic effect of the rifampicin treatment on *Rickettsia* populations.

Regarding *Francisella*-like endosymbiont, significant differences were observed in the ratios of concentration between the treatment groups with a decrease in the rifampicin group. When looking at the concentration of FLE, as observed for Rickettsia, the concentration of FLE tended to rise after blood meal in the gentamycin groups, whereas, it remained close to a basal level in the rifampicin one. More information was obtained from the qPCR performed on adult females following the two antibiotic treatments. We observed that the ratios of concentrations of both FLE and *Rickettsia* strongly decreased in the rifampicin groups and were slightly lowered in the gentamycin ones compared to the control groups 50 days after the second treatment.

Overall, the qPCR results suggest that the rifampicin treatments limited the growth and/or decreased the population size for *Rickettsia* and FLE. It seemed that the effect was bacteriostatic after one treatment on nymphs during growth. The diminution of the population of symbiont after two treatments can be the consequence of this bacteriostatic effect over time or the result of a bactericide effect after several consecutive treatments. In the end, the treatment performed was sufficient to prevent the growth of FLE and *Rickettsia* even though insufficient to eliminate completely the microbiota.

A slight effect was reported with the gentamycin treatment but this antibiotic (or, at least, the posology used for this antibiotic) seemed to be less effective than the rifampicin. Finally, no effect of the vitamin group was reported on the concentration of endosymbiont in adult ticks. This result was expected as the microbiota does not depend on B vitamins, it rather produces B vitamins.

Our experiments question the use of antibiotics during artificial blood feeding of ticks. In hard ticks, antibiotics and antifungal molecules are frequently added to the blood to avoid the development of micro-organisms during the long periods of times needed for the blood meal (Krull *et al*. 2017; Militzer *et al*. 2021; González, Bickerton, et Toledo 2021). This use of antibiotics and the molecules chosen should be carefully considered during experiment design as they will modify the tick's microbiota and, potentially, affects the ability of the tick to develop normally and to be maintained in a colony. This attention should also be increased for lab experiments performed to identify the potential influence of members of the microbiota on the presence of pathogens and *vice versa*.

Consequences of the modification of the microbiota on the tick physio-biology

In female ticks, considering only the antibiotic treatments, no effect was detected on the reproduction itself after the first treatment. This result would suggest that the FLE and *Rickettsia* are not implicated in the reproduction of the tick. However, at this point of the experiment it is also possible that the effect of treatment did not eliminate enough symbiont yet. Nevertheless, we observed that the negative control group presented a higher mortality rate than the antibiotic groups. This surprising result led us to consider the fitness cost of harboring endosymbionts, as already referenced for other arthropods (Ankrah, Chouaia, et Douglas 2018). For instance, it is possible to make the hypothesis that *Francisella* would harbor an essential long-term function for the tick (providing essential nutrients, avoiding a deficiency) but a short-term cost for the tick (increased mortality during reproduction). Another possible explanation would be that other bacterias than FLE and Rickettsia were eliminated by the antibiotic treatment and that those bacterias presented a fitness cost for the tick. This hypothesis remains less probable than a role of FLE and Rickettsia which are the most frequent in *O. moubata* (Duron *et al*. 2018).

Nevertheless, the effect of antibiotic treatments should not be considered alone. Several trends can be noticed for ticks which received vitamin supplementations. Vitamin supplementation tended to be associated with a reduction of the time between mating and first oviposition, an increase in the success rate of oviposition and, a slight increase in the success rate of the first molting. This is consistent with the idea that these vitamins

are essential for the tick and could lead to better reproductive and molting parameters and highlights the importance of nutritional symbionts such as FLE. However, these trends were not significant in our study and must be considered carefully. It is possible that the duration of our experiment was not sufficient to fully observe the effects of a vitamin deficiency. Moreover, FLE were maintained at a low concentration but not completely eliminated here, suggesting that all ticks were already supplied, at some extent, in B vitamins.

If we consider the results obtained after the second antibiotic treatment, two main elements can be stressed out. First, is the confirmation of an effect on tick survival. After both treatments, the control group presents a higher mortality than the gentamycin group. However, after the second treatment, the rifampicin group is also affected by an increased mortality rate. One hypothesis could be that more FLE have been eliminated after the second treatment and, as a consequence, the deficiency in B vitamin for the tick is stronger than in the first treatment, leading to the death of adult females. This hypothesis is comforted by the fact that the survival rate in the rifampicin group after the second treatment was better when the ticks were supplemented with B vitamins. From these results, it would seem that there is a cost to maintain high levels of symbionts (increased mortality in the control group), but the absence of symbionts results in a similar death rate (mortality in the second rifampicin group), and finally in the groups with moderate amount of symbiont, the benefits of endosymbiont compensate any associated fitness cost (gentamycin treatment groups).

Second, when regarding the parameters associated with reproduction, no clear-cut effect can be established after the second treatment. The trends noticed during first treatment for vitamin supplementation are not confirmed and the few significant results (egg incubation time and time to molting) present variations of low range. If it is difficult to interpret the variations for the egg incubation time, the variations on the molting time on the opposite are oriented with a longer time period for the rifampicin group. As suggested in the literature, endosymbionts are crucial during molting (Duron *et al*. 2018), and their elimination through rifampicin could indeed lead to a deterioration of the parameters associated with molting.

When looking at past literature on the role of endosymbionts for tick reproduction, several effects were associated with the elimination of *Coxiella*-like endosymbionts (CLE) in hard ticks, including a decrease in number of eggs laid (Guizzo *et al*. 2017; Zhang *et al*. 2017), an increase in the egg incubation time (Li, Zhang, et Zhu 2018), a decrease in the hatching rate (Guizzo *et al*. 2017; Li, Zhang, et Zhu 2018; Zhong, Jasinskas, et Barbour 2007) and a decrease in the molting success (Zhong, Jasinskas, et Barbour 2007). Interestingly, no effects were reported for *Rickettsia* (Kurlovs *et al*. 2014) or FLE. This is especially interesting as all these three endosymbionts have a nutritional role. The results presented here indicate no effect of FLE or *Rickettsia* on the tick reproduction. It is possible that CLE are the only tick symbionts which improve the reproductive fitness of their host in addition to B vitamin production, leading them to remain one of the most commonly found endosymbiont in ticks (Duron *et al*. 2017).

In our study, we presented results issued from two successive treatments. Interestingly, in *O. moubata*, the vitellogenesis processes start during the last nymphal stages (Sonenshine 1991) suggesting that the role of the microbiota on reproduction may occur before adult stage. In the future, antibiotic treatments will have to be performed starting from nymph stages to evaluate the role of the microbiota during these early stages on the reproduction of the adults obtained.

However, blood meal and mating are also critical steps for vitellogenesis processes (Horigane *et al*. 2010) and most studies performed on tick reproduction targeted directly adult females (Guizzo *et al*. 2017; Li, Zhang, et Zhu 2018; Zhang *et al*. 2017; Zhong, Jasinskas, et Barbour 2007; Kurlovs *et al*. 2014). Parameters such as the number of eggs laid depend directly on the efficiency of the blood meal. Moreover, as shown here and in previous studies (Sassera *et al*. 2008), endosymbiont load blooms after the blood meal strengthening the hypothesis that they have probably a role during and/or after the meal. Ideally, using germ-free tick lineages during blood meal would bring striking elements of response on this point. Such lineages are however difficult to sustain, and antibiotic treatment remains, for now, the main tool available.

Finally, even though it was not the objective of the study, monitoring adult females over two periods of egg-laying brought data on the effects of ageing on the reproduction. Similarly to results obtained in model invertebrates (Tatar 2010), the reproductive indicators in soft ticks were strongly deteriorated between the first and second period of egg-laying. Our results indicate no link between this phenomenon and the presence or absence of FLE and *Rickettsia* suggesting the effect is solely due to ageing.

To conclude, this study suggests that FLE and *Rickettsia* endosymbionts have no direct effect on the reproduction of their tick host. Several trends associated with these bacterial species were highlighted including a potential fitness cost, and strong load increase following blood meal.

However, elucidating the role of the tick microbiota remains an arduous task. Antibiotic treatments constitute a powerful tool for this aim, but protocols need to be adjusted and modified in each tick lineage to take into account the microbiota composition and sensitivity.

Author contributions

FT performed the experiments described, analyzed the data, and wrote the manuscript. TP provided the material, obtained the funding, and conceived and coordinated the study. MD provided the material and helped perform the experiments. LG helped perform the experiments and analyze the data. HJ provided the material, obtained the funding, conceived and coordinated the study, performed the experiments and analyzed the data. All authors read and corrected the final manuscript.

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Data, scripts and codes availability

Data and scripts will be available online on Dryad. Link and DOI will be added in version 2 of this preprint by Mai 26th.

Supplementary material

Table S1: Sample size and repartition of individuals into groups for the different experiments performed.

Experiment	Treatment group	Vitamin subgroup
Symbiont loads ; nymphs n=366	No treatment n=20	No treatment N=20
	Control n=116	Vitamin ; n=57
		No vitamin ; n=59
	Rifampicin n=110	Vitamin ; n=50
		No vitamin ; n=60
	Gentamicin n=120	Vitamin ; n=60
		No vitamin ; n=60
Symbiont loads ; females n=59	Control n=14	Vitamin ; n=7
		No vitamin ; n=7
	Rifampicin n=27	Vitamin ; n=13
		No vitamin ; n=14
	Gentamicin	Vitamin ; n=12

	n=18	No vitamin ; n=6
Reproduction monitoring ; first 50-days period n=132	Control n=46	Vitamin ; n=23
		No vitamin ; n=23
	Rifampicin n=42	Vitamin ; n=21
		No vitamin ; n=21
	Gentamicin n=44	Vitamin ; n=22
		No vitamin ; n=22
Reproduction monitoring ; second 50-days period n=99	Control n=27	Vitamin ; n=12
		No vitamin ; n=15
	Rifampicin n=33	Vitamin ; n=17
		No vitamin ; n=16
	Gentamicin n=39	Vitamin ; n=20
		No vitamin ; n=19

Note: Sample size variations between groups are the result of tick mortality in the group before the start of monitoring.

Table S2: Mean and variance for the traits associated with reproductive fitness after antibiotic treatment and vitamin supplementation.

Variable	First treatment	Second treatment
Laying probability	74.2% (98/132)	40.4% (40/99)
Survival over 50 days after blood meal	78.8% (104/132)	60.6% (60/99)
Number of days to first oviposition	23.9 days [SD: 6.81]	24.7 days [SD: 12.0]
Number of eggs / Number of eggs standardized by mother weight	109 eggs / 0.361 egg/mg [SD: 0.116]	65.2 eggs / 0.228 egg/mg [SD: 0.176]
Average egg mass	0.538 mg [SD: 0.0463]	0.516 mg [SD: 0.0671]
Egg incubation time	11.9 days [SD: 1.01]	12.4 days [SD: 1.18]
Egg hatching rate	79.6% [SD: 15.1]	55.0% [SD: 28.9]
Time for molting into first nymphal stage	6.30 days [SD: 0.657]	6.39 days [SD: 0.724]
Molting rate	98.4% [SD: 4.77]	98.5% [SD: 4.32]

Conflict of interest disclosure

The authors of this preprint declare that they have no financial conflict of interest with the content of this article.

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