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Spécialité : *Microbiologie, virologie et parasitologie*

Par
Romain DAVEU

Interactions entre la bactérie endosymbiotique *Candidatus* Midichloria mitochondrii et son hôte arthropode, la tique *Ixodes ricinus*

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DOCTORAL THESIS OF

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Research unit INRAE, Oniris, BIOEPAR, 44300 Nantes, France

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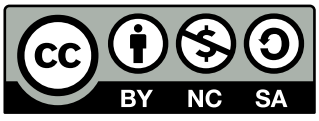

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For this section, I took the liberty to freely switch between English and French, so please, forgive me. And forgive my informal tone at times.

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Interactions entre la bactérie endosymbiotique *Candidatus* Midichloria mitochondrii et son hôte arthropode, la tique *Ixodes ricinus*

Introduction

Les tiques sont des ectoparasites hématophages responsables de la transmission de maladies importantes tant chez l'homme que chez l'animal (Jongejan et Uilenberg, 2004). Elles sont pour la plupart classées en deux grandes familles (tiques dures — Ixodidae — et tiques molles — Argasidae) présentant notamment des stratégies alimentaires très différentes. En particulier, environ 700 espèces de tiques dures se nourrissent de sang d'hôtes vertébrés trois fois au cours de leur vie, une fois pour chaque stade immature, larve et nymphe, pour atteindre l'âge adulte (Balashov, 1972 ; Guglielmone et al., 2014). Le dernier repas sanguin des femelles adultes dure environ 6-12 jours, et est nécessaire pour fournir des nutriments pour la production d'œufs (Balashov, 1972). *Ixodes ricinus* est la tique la plus répandue et importante en Europe, son aire de distribution s'étendant de la Scandinavie à l'Espagne, du Portugal à la Russie (Medlock et al., 2013). Cette espèce de tique est la plus étudiée en Europe en raison de son importance médicale, car les agents pathogènes qu'elle transporte, tels que *Borrelia burgdorferi* s.l., agent de la maladie de Lyme, et le virus TBE, responsable de l'encéphalite à tiques, sont capables d'être transmis à l'homme lors des repas sanguins des nymphes et des femelles adultes (Parola et Raoult, 2001b).

Outre les agents pathogènes, les tiques sont également les hôtes de bactéries symbiotiques transmises par la mère, dont il a été démontré qu'elles peuvent avoir des effets importants sur le phénotype de l'arthropode (Bonnet et al., 2017 ; Narasimhan et Fikrig, 2015). Dans certains cas, les symbiotes hérités de la mère sont devenus des mutualistes

obligatoires conservant ou acquérant la capacité de synthétiser des produits biochimiques importants essentiels à leurs hôtes arthropodes (Moran et al., 2008 ; Wernegreen, 2012).

Candidatus Midichloria mitochondrii (ci-après *Midichloria mitochondrii*) est l'endosymbiote le plus fréquent et le plus abondant d'*I. ricinus* (Aivelo et al., 2019 ; Guizzo et al., 2020 ; Sassera et al., 2006). Cette bactérie intracellulaire présente deux tropismes dans les ovocytes de son hôte : une partie de la population est cytoplasmique, tandis qu'une autre partie importante des symbiotes présente la particularité unique au sein du règne animal de pouvoir résider en grand nombre entre la membrane interne et la membrane externe des mitochondries des cellules de son hôte, changeant probablement de tropisme au cours de son cycle cellulaire (Lewis, 1979 ; Sacchi et al., 2004 ; Zhu et al., 1992). Ce tropisme intramitochondrial pourrait être considérer comme une interaction de type parasitisme ou de prédation de la mitochondrie. *Midichloria mitochondrii* présente un taux de fixation proche des 100% au sein des femelles adultes collectées sur le terrain, ainsi que chez les œufs et les larves (via une transmission verticale de la mère à la descendance) (Lo et al., 2006 ; Sassera et al., 2008). La connaissance du génome de la bactérie n'a pas pu apporter de conclusions claires quant à son rôle potentiel pour la tique, à part le possible apport de quelques vitamines B (biotine et folate) et d'autres co-facteurs pouvant être essentiels à son hôte (Sassera et al., 2011). Malgré tout, l'hypothèse d'un rôle mutualiste, bénéfique aussi bien pour la bactérie que pour la tique a été retenue, mais n'a pas pu être démontrée expérimentalement jusqu'alors (Ninio et al., 2015). Cependant la bactérie n'est détectée que dans environ la moitié des mâles — adultes —, et en très faible quantité par rapport aux femelles, questionnant l'apport par la bactérie de nutriments censés être essentiels à des réactions cellulaires basales, valables pour tous les stades de vie et indépendamment du sexe (Beninati et al., 2004 ; Lo et al., 2006 ; Sassera et al., 2008). La densité de *M. mitochondrii* au sein de la tique — femelle adulte — explose lors du gorgement, notamment dans l'ovaire (Olivieri et al., 2019 ; Sassera et al., 2008) et présente des densités plus modérées chez les stages immatures (larves et notamment nymphes). En plus de l'ovaire, les effectifs de *M. mitochondrii* semblent être non négligeables dans les tubes de Malpighi et les glandes salivaires, tout en étant inférieurs de plusieurs ordres de grandeur par rapports aux densités observées dans les ovaires (Olivieri et al., 2019). Les glandes salivaires sont des organes clés pour les pathogènes transmis aux vertébrés par les tiques. Ces glandes salivaires ont aussi un rôle majeur pendant le gorgement, où un cocktail de molécules est libéré à l'intérieur de l'hôte pour éviter la coagulation, la

sensation de douleur, les réponses inflammatoires ou contourner le système immunitaire de l'hôte vertébré, et donc permettre à la tique de continuer son repas sanguin en toute tranquilité (Sauer et al., 2000). Le fait de retrouver *M. mitochondrii* dans les glandes salivaires interrogent quant au fait de sa possible transmission aux hôtes vertébrés voire de son potentiel pouvoir pathogène pour les vertébrés (Cafiso et al., 2018 ; Mariconti et al., 2012 ; Serra et al., 2018). Cet endosymbiote pourrait aussi interagir directement ou indirectement avec des agents pathogènes et donc possiblement moduler la capacité vectorielle de son hôte arthropode (Budachetri et al., 2018). Les tubes de Malpighi sont considérés comme des organes importants pour la régulation osmotique, la détoxication et les fonctions excrétrices (production de déchets azotés), pouvant être apparentés à un rôle similaire aux reins des vertébrés, mais leur rôle fin dans la biologie des tiques reste mal caractérisé (Sonenshine et Roe, 2013). Dans le cas de certaines associations tique-symbiote (principalement avec des *Coxiella*-like endosymbiotes et des *Francisella*-like endosymbiotes), les tubes de Malpighi ont été rapportés comme étant un organe clé pour accueillir le symbiote, où des agrégats de symbiotes se forment, qui pourraient être comparés à des bactériocytes d'insectes (Buysse et al., 2019 ; Duron et al., 2018 ; Guizzo et al., 2017 ; Klyachko et al., 2007).

La caractérisation de la relation entre un bioagresseur et son ou ses endosymbiotes principaux est d'intérêt majeur car elle permet parfois de mettre en évidence le caractère obligatoire de la symbiose entre l'hôte et la bactérie, et donc d'exploiter cette dépendance dans le cadre d'une lutte antivectorielle basée sur un contrôle antisymbiotique par exemple. Un des cas les mieux connus est probablement l'utilisation d'antibiotiques contre le nématode parasite responsable de la filariose de Malaisie, *Brugia malayi*, qui a comme impact de supprimer la bactérie endosymbiotique *Wolbachia* dont il dépend de façon obligatoire (Bandi et al., 1999 ; Ru, 2005).

Objectifs de la thèse

La présente thèse se divise en trois chapitres principaux qui ont pour but de répondre à différentes questions concernant les interactions entre *M. mitochondrii* et *I. ricinus*.

- L'hypothèse a été soulevée que les nymphes femelles étaient déjà hôtes de davantage de bactéries comparés aux nymphes mâles. Ainsi pour étudier la dynamique

de *M. mitochondrii* dans ce stade sexuellement immature d'*I. ricinus*, un design expérimental a été conçu pour inférer le sex-ratio de groupe de nymphes (la détermination du sexe n'étant pas possible en l'absence de dimorphisme sexuel et de marqueur génétique) dont la densité de *M. mitochondrii* a été systématiquement étudiée.

- La création d'une lignée de tiques dépourvues de symbiotes et son témoin hébergeant le symbiote permet des comparaisons de traits d'histoire de vie de la tique afin de caractériser l'impact de la présence ou absence du symbiote sur la valeur adaptative de son hôte. C'est ce qui a donc été tenté via l'injection d'antibiotiques dans des femelles non-gorgées. La densité des symbiotes dans la descendance a été par la suite caractérisée. A l'instar d'autres symbiotes qui en ont la capacité (Duron et al., 2018 ; Nardi et al., 2021 ; Gottlieb et al., 2015 ; Gerhart et al., 2016), l'hypothèse d'un apport de vitamines B par *M. mitochondrii* à *I. ricinus* a été testée en parallèle.

- L'étude de l'impact du symbiote sur l'expression des gènes des tiques dans le but de mieux comprendre l'effet de *M. mitochondrii* sur son hôte. De ce fait, le transcriptome d'*I. ricinus* avec et sans le symbiote a été étudié, au sein de plusieurs organes, ce qui est une première chez les tiques. Des gènes de la tique différentiellement exprimés en réponse à la présence du symbiote, et potentiellement impliqués dans la relation avec celui-ci, ont été caractérisés.

Chapitre I - La dynamique des symbiotes pendant le repas sanguin des nymphes d'*Ixodes ricinus* diffère selon leur sexe

Avant-propos

Cette étude, publiée dans la revue *Ticks and Tick-Borne Diseases* en mars 2021, vise à décrire la dynamique de la charge en *M. mitochondrii* des nymphes d'*I. ricinus* suite à leur engorgement. Ces résultats apportent de nouvelles connaissances sur la charge en *M. mitochondrii* d'un stade sexuellement immature d'*I. ricinus* (stade nymphal), avant la multiplication importante de l'endosymbiote dans les gonades des femelles matures suite

à un repas sanguin (Sassera et al., 2008). Ces connaissances sont donc utiles pour mieux apprécier le rôle de *M. mitochondrii* sur la biologie de son hôte arthropode en se concentrant sur une phase du cycle du parasite permettant d'explorer des changements liés à son ontogenèse. Ce travail trouve son origine dans le stage de Cindy Laurence (étudiante en 2upe année de Master en 2014 encadrée par Olivier Plantard). Pour cet article, j'ai réanalysé l'ensemble des données qui ont pu être acquises et mené des analyses statistiques poussées pour finalement rédiger un manuscrit décrivant et discutant les résultats obtenus.

Contexte

Après avoir conçu un protocole de qPCR permettant la quantification de *M. mitochondrii*, Sassera et al., 2008 ont étudié la dynamique de la charge en *M. mitochondrii* à travers tous les stades de vie des tiques (de l'œuf à l'adulte, en passant par les deux stades intermédiaires larves et nymphes) à la fois non-gorgées et gorgées. Outre le fait que la quantité absolue de bactéries symbiotiques entre les femelles et les mâles adultes était très différente, un résultat notable de cette étude était la grande variance de la densité des symbiotes observée chez les nymphes gorgées. Cette dernière observation avait déjà été interprétée comme suggérant "une spécialisation du symbiote vers les femelles au stade nymphal". Cependant, l'interprétation de cette forte variance de la densité des symbiotes parmi les nymphes était limitée par le fait que les auteurs "n'étaient pas en mesure de déterminer le sexe des (larves et) nymphes". Ainsi, l'étude décrite ici a été spécifiquement conçue pour évaluer avec davantage de précision la densité des symbiotes dans des lots de nymphes présentant des sexe-ratios différents (étant donné qu'aucun marqueur génétique du sexe chez *I. ricinus* n'est disponible à ce jour).

Méthodes

Deux cent cinquante nymphes sauvages non gorgées d'*I. ricinus* ont été collectées en janvier 2014 dans la forêt de Chizé, France. Trois lots de nymphes non gorgées ont été constitués sur la base de caractères morphométriques longueur du scutum et de l'hypostome), avec l'hypothèse que (i) le premier groupe avec les plus petites valeurs morphométriques était principalement constitué de mâles, (ii) le deuxième groupe avec les longueurs intermédiaires de l'hypostome et du scutum correspondait à un sexe-ratio équilibré, (iii)

le troisième groupe avec les plus grandes valeurs morphométriques était principalement constitué de femelles. Les nymphes ont été pesées avant le gorgement, puis une partie a été sacrifiée afin de déterminer la densité de *M. mitochondrii* tandis que le reste a été déposé sur un hôte vertébré pour réaliser son repas sanguin. Après le repas, les nymphes gorgées ont été pesées à nouveau, puis une partie a été mise de côté pour permettre de muer en adulte, tandis que le reste a été sacrifié pour extraire son ADN et mesurer la densité de *M. mitochondrii* par qPCR.

Résultats principaux

Alors qu'aucune différence significative dans la masse corporelle ou la charge en *M. mitochondrii* n'a été observée au stade non gorgé, ces deux caractéristiques étaient significativement différentes entre chaque groupe de taille après le gorgement.Nos résultats démontrent que la dynamique des symbiotes pendant le gorgement des nymphes est bien différente entre les deux sexes. Ainsi, une charge de *M. mitochondrii* significativement plus élevée chez les nymphes qui deviendront des femelles est observée. Ces résultats suggèrent que l'augmentation de la taille du primordium ovarien (chez les nymphes qui deviendront des femelles) suite au repas sanguin est un processus clé pour expliquer la forte multiplication de *M. mitochondrii* pendant cette partie du cycle de vie de la tique.

Bien qu'il existe un nombre croissant d'articles étudiant la dynamique des endosymbiotes après un repas sanguin chez les femelles de diverses espèces de tiques (Oliver et al., 2020 ; Wang et al., 2018), il s'agit, à notre connaissance, de la seule étude étudiant la dynamique des symbiotes chez les nymphes en fonction de leur sexe bénéficiant d'une telle précision.

Le développement des investigations en cours sur la génomique des tiques (Jia et al., 2020) et la quantité croissante de ressources associées (Murgia et al., 2019) devraient bientôt fournir des marqueurs du chromosome Y (présent chez les tiques mâles uniquement) et permettre ainsi d'étudier la charge en *M. mitochondrii* chez des nymphes individuelles de sexe connu de façon encore plus précise et même dans des stades encore plus précoce comme les larves ou les oeufs.

Chapitre II - Antibiotiques et injections de vitamines B : tentative d'élimination de *M. mitochondrii*et supplémentation nutritionnelle

Ce chapitre nécessite une refonte expérimentale ainsi que l'obtention d'un échantillonnage de tiques plus important pour permettre de tirer des conclusions biologiques et n'a pas à vocation à être publié dans un journal sous sa présente forme.

Avant-propos

Pour étudier le rôle d'un symbiote sur la biologie de son hôte arthropode, les comparaisons entre des souches aposymbiotiques (dépourvues de leurs symbiotes) et sauvages (sans modification/réduction du microbiote naturel) de diverses caractéristiques biologiques (traits d'histoire de vie tels que fécondité ou espérance de vie, survie après exposition aux pathogènes/parasites, reproduction, nutrition et taux de développement) sont particulièrement utiles. La disponibilité de telles souches aposymbiotiques a eu une importance majeure dans la compréhension des interactions complexes et diversifiées observées entre les symbiotes et leur hôte (Oliver et al., 2009a). Cependant, la production de telles souches aposymbiotiques peut parfois se révéler être une tâche particulièrement ardue. C'est notamment le cas pour les tiques, arthropodes strictement hématophages, présentant un cycle de vie long (de deux à trois ans dans la nature) et nécessitant trois repas sanguins différents sur trois individus-hôtes différents pour achever leur développement et produire une descendance. Bien que le recours à des antibiotiques soit un outil fréquemment utilisé pour supprimer les symbiotes de leurs hôtes arthropodes, il s'avère plus délicat dans le cas de *M. mitochondrii*. En effet, cette bactérie ne pouvant pas être cultivée sur des milieux artificiels (il n'existe à ce jour aucune lignées cellulaires de tiques hébergeant cette bactérie ; les lignées décrites dans Najm et al., 2012 hébergeant une bactérie proche de *M. mitochondrii* qui n'est pas à strictement parler la même ; Sassera, communication personnelle) et la tique étant un hôte avec un long cycle de vie, obligeant les expérimentateurs à s'armer de patience pour voir les effets d'un traitement, il est particulièrement difficile de tester différents antibiotiques afin de sélectionner le plus efficace. De plus, l'utilisation de systèmes de gorgement artificiel pour nourrir les tiques s'avère difficilement compatible avec la disponibilité d'une lignée de tiques témoin (hébergeant le symbiote d'intérêt). En effet, si des antibiotiques ne sont pas utilisés au sein de ces gorgeurs artificiels, la

contamination du sang est difficilement évitable au sein du système (sur la membrane par exemple) qui doit être conservé pendant les unes ou deux semaines que durent le gorgement des tiques, dont le repas sanguin est beaucoup plus long que chez les autres arthropodes hématophages (Bonnet et Liu, 2012). En général, le taux de réussite du repas sanguin sur gorgeur artificiel est aussi plus faible et sa durée est plus longue par rapport au gorgement sur un hôte vivant (Militzer et al., 2021). De plus, pour les symbiotes obligatoires — une caractéristique qui peut être considérée comme compatible avec la prévalence observée de presque 100% de *M. mitochondrii* chez les femelles sauvages d'*I. ricinus* —, on s'attend à ce que les souches de tiques aposymbiotiques ne puissent pas survivre (ou difficilement), à moins qu'une supplémentation correspondant à l'apport métabolique du symbiote ne soit fournie (en ajoutant des vitamines B dans le régime alimentaire par exemple ; Duron et al., 2018 ; Hosokawa et al., 2010).

Objectifs

Le premier objectif de ce travail était la production d'une souche aposymbiotique d'*I. ricinus* dépourvue de *M. mitochondrii* via l'injection de l'antibiotique tétracycline (notamment pour une utilisation ultérieure au chapitre III basée sur la comparaison de lignée de tiques avec et sans symbiote). Par ailleurs, afin de tester si *M. mitochondrii* fournit bien des vitamines B à son hôte arthropode (comme certains autres endosymbiotes se sont avérés l'être, ou sont suspectés de l'être de part leurs capacités génomiques), une partie des tiques ont été nano-injectées à la fois avec des vitamines B et de la tétracycline (en même temps que des tiques avec seulement des vitamines B, à des fins de contrôle). Deux concentrations d'antibiotiques ainsi que deux concentrations de vitamines B ont été testées, donnant lieu par la combinatoire à l'étude de neuf traitements (en incluant le contrôle d'injection d'eau). Divers traits d'histoire de vie des tiques ont été comparés entre ces différents traitements pour évaluer l'implication de *M. mitochondrii* dans leur biologie et son potentiel rôle nutritionnel.

Résultats principaux

Après injection des différents traitements dans des femelles non gorgées d'*I. ricinus*, un taux de mortalité anormalement élevé et inattendu a été observé au bout de quelques jours, en particulier pour les traitements antibiotiques et vitamines B avec les concentrations les plus élevées. Ce taux de mortalité a réduit de façon drastique la taille de

l'échantillon analysable, empêchant toute conclusion statistique potentiellement significative. Parmi les traits d'histoire de vie mesurés, la masse de femelles gorgées et la masse d'œufs semblent être inférieures par rapport au contrôle et aux autres traitements pour le traitement antibiotique fortement dosé. Pour autant, aucune perte apparente ou variation de densité de *M. mitochondrii* n'a été observée dans la descendance de ces femelles dans lesquelles des antibiotiques ont été injectées.
Plusieurs problèmes méthodologiques (ayant pu nuire à la survie des tiques ou à l'élimination de *M. mitochondrii*) nous ont empêchés de tirer toute conclusion sur l'effet phénotypique de la présence de *M. mitochondrii* sur *I. ricinus*. Afin d'augmenter les chances de succès d'une telle expérience, il serait utile de tester une localisation différente pour l'injection conjointement à l'utilisation (i) d'un micromanipulateur pour améliorer la précision de l'injection, (i) d'un antibiotique différent tel que la ciprofloxacine (en comparaison avec plusieurs autres), la ciproflaxine s'étant révélée plus efficace que la tétracycline pour réduire la densité d'un endosymbiote de tique phylogénétiquement proche de *M. mitochondrii* (*Rickettsia* endosymbiote d'*I. pacificus* ; Kurlovs et al., 2014). Par ailleurs, la dose la plus faible pour le mélange de vitamines B (0.1X) permettrait de prévenir toute mort prématurée excessive des tiques due aux propriétés chimiques (par exemple : pH, molarité) des différents traitements et démontrer ainsi le rôle de *M. mitochondrii* sur les tiques et la possible supplémentation nutritionnelle par les vitamines B.

Chapitre III - Comparaison du niveau d'expression des gènes d'*Ixodes ricinus* au sein de différents organes en fonction du statut symbiotique

Manuscrit en préparation (à soumettre sous une forme modifiée et plus concise).

Avant-propos

Les endosymbiotes d'arthropodes sont connus pour avoir un impact sur de nombreuses fonctions biologiques de leur hôte, dont notamment la nutrition (et donc le développement), la reproduction ou la réponse immunitaire (Haine, 2008 ; Moran et al., 2008 ; Welchman et al., 2009 ; Wernegreen, 2012). Pour identifier les fonctions biologiques influencées par la présence ou l'absence d'un symbiote, une approche transcriptomique

est particulièrement pertinente car elle permet une investigation intégrative prenant en compte simultanément tous les gènes (ou transcrits) exprimés par l'organisme hôte. La question principale à l'origine de ce travail était de caractériser la réponse transcriptomique de *I. ricinus* en présence ou en absence de *M. mitochondrii*, afin de mieux comprendre l'interaction entre l'hôte et le symbiote. Afin de réduire la complexité du système et de faciliter l'interprétation des données sur l'expression différentielle des gènes, nous avons décidé de considérer séparément trois organes différents :

- l'ovaire qui est l'organe où *M. mitochondrii* présente la plus forte densité et donc un organe clé pour comprendre les interactions entre cette bactérie et son hôte arthropode (Beninati et al., 2004 ; Olivieri et al., 2019 ; Sacchi et al., 2004).

- les tubes de Malpighi qui sont connus pour abriter certains endosymbiotes qui se sont révélés être des symbiotes obligatoires chez d'autres espèces de tiques (Buysse et al., 2019 ; Duron et al., 2018 ; Klyachko et al., 2007) et où *M. mitochondrii* a été détecté mais en quantité nettement moindre (Olivieri et al., 2019).

- les glandes salivaires qui sont les organes les mieux connus pour la transcriptomique des tiques (par exemple Garcia et al., 2014 ; Martins et al., 2019 ; Perner et al., 2018 ; Ribeiro et Mans, 2020 ; Schwarz et al., 2013), permettant une comparaison avec des études antérieures, y compris le changement d'expression génique dû à la présence de bactéries pathogènes particulières.

De plus, comme le repas sanguin est connu pour avoir un effet considérable sur l'expression de nombreux gènes de tiques (en lien avec le développement important de ces trois organes après l'alimentation) (Karim et Ribeiro, 2015 ; Rudenko et al., 2005 ; Sonenshine et Roe, 2013) et sur l'explosion de *M. mitochondrii* (Sassera et al., 2008), cette étude transcriptomique a été menée à la fois sur des femelles *I. ricinus* non alimentées et partiellement alimentées.

Même s'il existe des projets de séquençage du génome entier de tiques en cours ou publiés (Gulia-Nuss et al., 2016 ; Jia et al., 2020 ; Murgia et al., 2019), l'annotation

fonctionnelle des gènes de tiques est encore limitée et aucun génome annoté d'*I. ricinus* n'est actuellement disponible. Pour cette raison, j'ai dû assembler *de novo* un transcriptome pour les organes d'*I. ricinus* étudiés ici, en utilisant les jeux de données générés pour cette étude.

Contexte

Etant donné la relation intime dans laquelle *I. ricinus* et *M. mitochondrii* sont associés, la première difficulté à laquelle nous avons dû faire face a été d'obtenir une souche aposymbiotique d'*I. ricinus*, exempte de *M. mitochondrii*, ou au moins avec un titre significativement réduit de la bactérie. Une autre difficulté était de minimiser les différences entre les souches symbiotiques et aposymbiotiques pour évaluer avec un maximum de confiance que les effets observés sur le transcriptome étaient dus à la présence ou à l'absence du symbiote. Pour cela, il est nécessaire que les différences génétiques et de statut physiologique (en lien avec les conditions environnementales) soient aussi faibles que possible entre les deux souches. La voie la plus directe serait de collecter une souche sauvage d'*I. ricinus* hébergeant *M. mitochondrii* et de retirer le symbiote pour la moitié de ces tiques. Dans ce but, nous avons choisi d'effectuer des injections d'antibiotiques. Comme nous l'avons vu au chapitre II, peu de choses sur l'efficacité des antibiotiques contre le genre *Midichloria* sont connues. La tétracycline a été testée sur des femelles *I. ricinus* engorgées mais n'a pas conduit à une réduction notable de l'endosymbiote (Ninio et al., 2015, Chapitre II). Quelques autres tentatives ont été essayées au laboratoire avant le début de ma thèse, notamment avec la marbofloxacine. Des femelles non-gorgées F0 collectées dans la nature ont ainsi été injectées soit avec de la marbofloxacine (MARB), soit avec une solution saline phosphatée 1X (PBS, comme contrôle) dès octobre 2015. Malheureusement, des difficultés de gorgement des nymphes et un taux de mortalité élevé lors de leur métamorphose en adulte ont conduit à ce qu'il ne reste plus qu'une seule femelle F1 MARB en février 2018. Pour obtenir un nombre suffisant de femelles, une autre génération F2 a été lancée. La durée du cycle de vie des tiques peut être raccourcie dans des conditions de laboratoire par rapport au cycle de vie dans la nature, cependant nous avons été confrontés à d'importants retards dans le temps de métamorphose des nymphes gorgées par rapport à ce à quoi nous étions habitués (quatre à six mois au lieu d'un). Pendant ce temps, la densité de *M. mitochondrii* a été quantifiée à chaque stade de développement selon la méthode décrite dans Sassera et al., 2008. La lignée MARB a été confirmée comme ayant une

quantité indétectable d'endosymbiote et la lignée PBS a maintenu un titre comparable de *M. mitochondrii* par rapport à ce qui a été décrit dans la littérature (Sassera et al., 2008).

La création d'une lignée d'*I. ricinus* dépourvue de *M. mitochondrii* semble donc faisable, indiquant que la relation présumée obligatoire entre *I. ricinus* et ce symbiote doit être traitée avec prudence, malgré d'apparent coûts importants sur la fitness de cette lignée aposymbiotique comme en atteste les retards de développement et les fortes mortalités.

Comme nous n'avons pas pu produire une nouvelle lignée de tiques après l'injection d'antibiotiques (chapitre II) et que la lignée aposymbiotique (MARB) et sa lignée témoin (PBS) produites au laboratoire n'étaient pas disponibles en nombre suffisant à temps pour réaliser l'expérience, nous avons utilisé une ancienne souche de laboratoire (plus de 30 générations au laboratoire) de l'Université de Neuchâtel, Suisse, aimablement fournie par le Dr. Maarten Voordouw. Cette souche a perdu *M. mitochondrii* au fil des générations. Le phénomène de perte progressive de *M. mitochondrii* dans les souches de laboratoire au fil des générations a déjà été mentionné dans la littérature (Lo et al., 2006). Pour les besoins de ce travail, cette souche de laboratoire a été comparée à une souche sauvage hébergeant *M. mitochondrii*, collectée à Chizé, France.

Méthodes

Les trois organes étudiés (à savoir l'ovaire, les tubes de Malpighi et les glandes salivaires) ont été disséqués avec précaution soit après l'accouplement sans aucun engorgement (point de temps J0) soit quatre jours après l'accouplement et le dépôt sur l'hôte vertébré (point de temps J4 — gorgement partiel) pour les deux souches (à savoir WT — pour les tiques sauvages de Chizé, France — ou Lab — pour la souche de laboratoire provenant de Neuchâtel, Suisse). Les ARN totaux de chaque échantillon ont été extraits et envoyés à la société Novogene, Hong-Kong, pour le séquençage de l'ARN par sélection poly(A). Les lectures brutes obtenues ont été coupées et leur qualité a été contrôlée. Un assemblage de méta-transcriptome a été construit à partir de six assemblages indépendants (par organe et point de temps) et annoté par rapport à différentes bases de données. L'expression des gènes a été évaluée après alignement et comptage des lectures par rapport aux différents transcrits. Une analyse exploratoire des librairies a été réalisée. Une analyse d'expression différentielle a été réalisée pour chaque tissu indépendamment, en comparant les stades

d'alimentation et le statut symbiotique. Cette étape a été suivie d'analyses d'enrichissement des termes de Gene Ontology (GO) et de tests d'ensembles de gènes pour évaluer les modèles fonctionnels. Les gènes significativement exprimés de manière différentielle avec des logs fold-changes extrêmes ont été occasionnellement considérés individuellement, notamment lorsqu'aucune association de termes GO n'a pas été trouvée (qui correspondait à environ 50% des gènes de l'assemblage).

Résultats et discussion

Les métriques mesurées indiquent une excellente qualité de l'assemblage du transcriptome de par le nombre de gènes BUSCO trouvés dans l'assemblage. Le regroupement par ACP des différentes librairies est d'abord observé en fonction des organes suivi par le statut d'alimentation (partiellement gorgé ou non). Ce n'est que lorsque chaque organe est considéré individuellement que les libraries se regroupent par statut d'alimentation et par statut symbiotique.

Globalement, les analyses d'expression différentielle ont révélé beaucoup plus de gènes différentiellement exprimés pour les comparaisons du statut d'alimentation par rapport aux comparaisons du statut symbiotique (environ 10 fois plus). En ce qui concerne les comparaisons du statut d'alimentation, parmi les termes GO, lorsqu'on considère l'ontologie GO "Processus Biologique" (BP, correspondant aux "transformations moléculaires nécessaires au fonctionnement d'entités biologiques intégrées"), le terme "Protein phosphorylation" est systématiquement enrichi pour les tiques partiellement gorgées, quel que soit l'organe considéré et indépendamment du statut symbiotique. De plus, pour la première fois, ce travail a permis de caractériser les principales fonctions biologiques enrichies dans les tubules de Malpighi à deux moments du cycle de vie de la tique (comparaison non-gorgée et gorgée). En particulier, les gènes codant pour plusieurs sulfotransférases au stade non gorgé sont ceux qui montrent les logs fold-changes les plus élevés. Concernant l'ontologie GO "Fonction Moléculaire" (MF ; correspondant aux "fonctions moléculaires réalisées, par exemple structurelles ou catalytiques"), le terme GO associé ("Sulfotransferase activity") est enrichi de manière particulièrement significative. L'ovaire, lors du milieu du gorgement, décrit un enrichissement pour les rôles biologiques notamment liés à la transduction du signal et aux réponses qui pourraient être interprétées comme la préparation de changements morphologiques dramatiques qui vont survenir lors de la dernière phase rapide du gorgement.

En ce qui concerne les comparaisons du statut symbiotique, environ deux fois plus de gènes sont exprimés de manière différentielle à J0 par rapport à J4. La présence de *M. mitochondrii* dans l'ovaire à mi-gorgement provoque un enrichissement des catégories BP GO se référant à "activation of NF-kappaB-inducing kinase activity" et "positive regulation of JNK cascade", ce qui pourrait être potentiellement interprété comme un déclenchement du système immunitaire inné de la tique, notamment par l'activation de six gènes indéterminés annotés en tant que "RING-type domain-containing protein" ou "Putative TNF receptor-associated factor" (TRAF). Les TRAF sont notamment des composants intermédiaires de la voie Toll (Fogaça et al., 2021). Cette voie pourrait provoquer la synthèse de peptides antimicrobiens (AMP). Un gène codant pour un AMP annoté comme "ricinusin" était le gène le plus ou l'un des plus différentiellement exprimés en termes log fold-change en présence de *M. mitochondrii* quelle que soit la comparaison considérée, interrogeant quant au lien existant entre la bactérie et ce peptide antimicrobien. Toujours dans l'ovaire mais au stade non gorgé, l'enrichissement du terme GO MF "heme binding" dans la souche sauvage (portant donc *M. mitochondrii*), pourrait être à mettre en lien avec la biosynthèse de l'hème par *M. mitochondrii* (Sassera et al., 2011). L'hème, toxique à haute dose, provient majoritairement du repas sanguin et les tiques n'ont pas la capacité de le synthétiser (Perner et al., 2016b). Ce cofacteur est stocké dans des organites spécialisés (hémosomes, au sein de cellules intestinales ; Lara et al., 2003) et utilisé avec parcimonie lors des besoins cellulaires (Lara et al., 2005). Il n'est donc pas exclu que *M. mitochondrii* puisse fournir une quantité non négligeable de ce cofacteur dans les ovaires pendant les stades non gorgés d'*I. ricinus*. En outre, le terme GO Cellular Component (CC) "TIM22 mitochondrial import inner membrane insertion complex" était enrichi pour les points de temps non gorgés et partiellement gorgés pour l'ovaire de la lignée symbiotiques. Les deux gènes liés à ce terme sont annotés comme "Mitochondrial import inner membrane translocase subunit Tim29" et sont potentiellement des cibles pour comprendre les subtilités liées au tropisme intramitochondrial particulier de *M. mitochondrii*. Même si ces hypothèses doivent être explorées expérimentalement, ces nouvelles données transcriptomiques permettent d'apporter de nouvelles pistes quant à l'implication de ces différents gènes/voies de biosynthèse dans les interactions *M. mitochondrii–I. ricinus*.

Conclusion

La nature des interaction entre *M. mitochondrii* et son hôte arthropode semble être plus complexe par rapport à d'autres endosymbiotes de tiques tels que *Francisella*-like endosymbiote et *Coxiella*-like endosymbiote (Duron et Gottlieb, 2020). Des observation suggèrent même que *Francisella* aurait pu remplacer *Coxiella* dans son rôle d'apport de vitamines au cours de l'évolution, si besoin est (Binetruy et al., 2020). Par ailleurs, alors que les autres genres de tiques présentent généralement une association privilégiée avec un seul genre d'endosymbiote, dans le cas du genre *Ixodes*, la situation semble plus complexe (Duron et al., 2018). Chez *I. ricinus*, une attention particulière a été portée sur *M. mitochondrii* qui se révèle être l'endosymbiote le plus commun de cette espèce (Aivelo et al., 2019 ; Guizzo et al., 2020 ; Lejal et al., 2020a ; Lejal et al., 2020b). D'autres endosymbiotes hérités maternellement sont présents chez *I. ricinus*, certainement avec une prévalence moindre mais ne doivent pas être négligés, ni le reste des espèces constituant le microbiote associé à cette tique. D'une part, *Rickettsia* sp. et *Spiroplasma ixodetis* (Duron et al., 2017 ; Lejal et al., 2020b ; Sprong et al., 2009) sont probablement héritées maternellement, tandis que la présence de *Wolbachia* et d'*Arsenophonus* est probablement causée par le parasitisme de l'hyménoptère parasitoïde *I. hookeri* (Bohacsova et al., 2016 ; Plantard et al., 2012). Les interactions complexes entre toutes ces espèces bactériennes font encore l'objet de recherches, où chacune pourrait jouer un rôle différent, avec certaines pouvant être impliquées dans de réelles relations de type mutualiste. Cependant, comme cela est de plus en plus fréquemment rapporté chez d'autres animaux, y compris les arthropodes, *I. ricinus* pourrait n'être que partiellement ou facultativement dépendant de son microbiome (Hammer et al., 2017 ; Hammer et al., 2019).

L'éventuel mutualisme de *M. mitochondrii* n'a donc pour l'instant pas été formellement et expérimentalement prouvé ou réfuté. Et pourtant, le tropisme intramitochondrial unique qui se produit chez *I. ricinus* et certaines autres espèces de tiques (Beninati et al., 2004, Floriano et al., en préparation), qui pourrait être interprété comme un comportement parasitaire, ne dépend pas de la caractéristique d'association presque fixée au sein d'une espèce (Beninati et al., 2009 ; Gofton et al., 2015). Par ailleurs, différentes espèces de *Midichloria* spp. étroitement liées à la seule espèce décrite (*M. mitochondrii* souche IricVA) pourraient présenter des associations symbiotiques différentes avec leur hôte (Buysse et Duron, 2018 ; Cafiso et al., 2016). Placer *M. mitochondrii* dans une case bien délimitée n'est donc pas chose aisée.

INTRODUCTION

Microbiome

The term microbiome has been first defined by Whipps et al., 1988 as "a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties", the concept encompasses not only the microorganisms involved in the interplay, but also "their theatre of activity". This definition is refined by Berg et al., 2020, by adding that the so-called "theatre of activity" results in the formation of specific ecological niches. Thus, the microbiome can be seen as a dynamic and interactive interaction, with a micro-system integrated within a macro-system, evolving upon time and scale (Berg et al., 2020, Fig. 1). The term microbiome has to be distinguished from the term microbiota which represents all organisms involed in a microbiome, independantly of the kingdom, whether Prokaryotes, Eukaryotes or viruses and derivatives — although the inclusion of viruses is under debate, and could be refer to the virome (Berg et al., 2020, Fig. 1).

The term holobiont refer to the ensemble between a host and the many other species living in or around it — microbiome, virome and other —, which together form a single ecological unit (Zilber-Rosenberg and Rosenberg, 2008; Theis et al., 2016). Hence, the organisms forming the whole supra-organism form a symbiosis. However, other authors suggest that the holobiont concept cannot be applied to host-microbiome symbioses, but should be better defined as "an ecological community of organisms that encompasses a broad range of interactions (parasitic to mutualistic), patterns of transmission (horizontal to vertical), and levels of fidelity among partners" (Douglas and Werren, 2016).

Endosymbiosis and endosymbionts

The study of symbiosis lasts back to the 19$^{\text{th}}$ century. Indeed, Heinrich Anton de Bary studied the formation of a well-known association between a fungi and an alga, the lichens. In the late 1870s, his work led him to coin the term "symbiosis" to define the concept of the "living together of unlike organisms" (De Bary, 1879). The term suggests that the

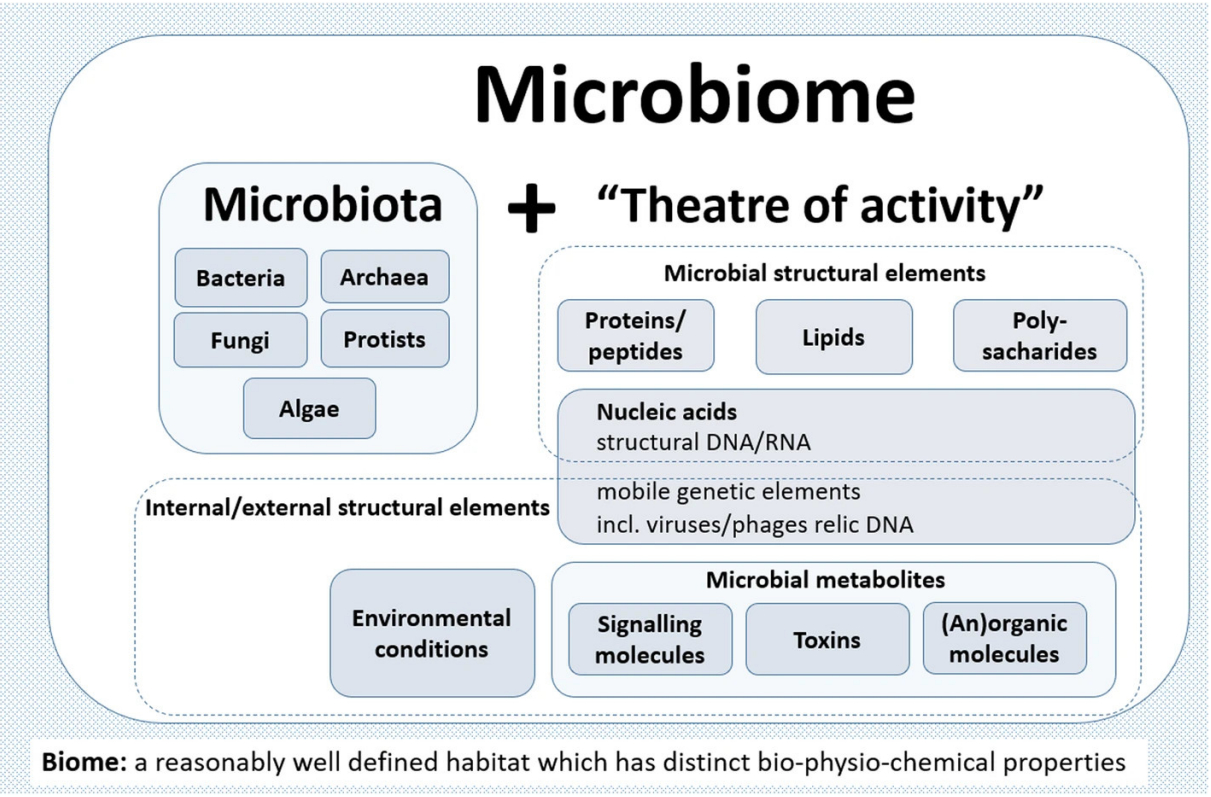

Figure 1 – **A schematic highlighting the composition of the term microbiome containing both the microbiota (community of microorganisms) and their "theatre of activity" (structural elements, metabolites/signal molecules, and the surrounding environmental conditions)** (from Berg et al., 2020).

interaction is beneficial to both organisms, which has long been a source of debate (Martin and Schwab, 2012). However, a broader definition allows for the inclusion of any intimate interaction between (at least) two partners, spanning a continuum from parasitism — deleterious to one at the expense of the other — through commensalism — beneficial to the commensal but neutral to the fitness of the host — to mutualism — advantageous to both (Moran, 2006). A given interaction may fall anywhere on this spectrum, but it may also be more complex and not entirely fit into one of these definitions.

The actors of a symbiosis are named symbionts. Indeed, in the case of the association between two organisms, both organisms (or "bionts") could be called symbionts. However, we traditionally choose to define symbiont as being the smallest organism, harboured by a host. Thus, endosymbiosis refers to the fact that a symbiont lives within another organism (Wernegreen, 2012). This biological interaction appears to be widespread among the tree of life.

Within arthropods, symbiosis is notably found for animal feeding on a nutrient skewed diet. Sap-sucking arthropods — due to their prominent role as pests in agriculture, either directly or through plant–pathogen transmission — and blood-sucking arthropods — due to their prominent role as vectors of infectious agents for human or animals — have received the most attention to date. However, the use of molecular methods have allowed to expand the investigation to various groups of organisms where symbiosis proved to be a major process both for the ecology and the evolution of life.

Primary symbionts

Many insects and other arthropods rely on intracellular microbial symbionts to provide nutrients such as essential amino acids and vitamins that they are not able to synthesise (Douglas, 1989). This type of symbiosis is referred to as mutualism. However, the beneficial effect appears more evident on the host than on the symbiont even if the host provides a house for the symbiont, which cannot grow outside host cells or body (Keeling and McCutcheon, 2017). Apart from the mutualist–parasite spectrum, endosymbionts can be divided into two arbitrary categories, primary and secondary endosymbionts. Typically, the primary endosymbionts are considered in the entomology world as (i) obligate, meaning their mutual interaction is so beneficial that the host cannot survive without the

symbiont (which is also the case for the symbiont by extension), (ii) bacteriome bound, i.e. the symbionts are usually confined in dedicated organ of the host body where nutrient exchange occurs and (iii) vertically inherited from the mother to the progeny, ensuring that the host–symbiont relationship continues from generation to generation (Boyd et al., 2014; Bright and Bulgheresi, 2010). This last point sometimes leads to a congruence of host and symbiont phylogenies, indicating that the vertical transmission has indeed been going on for a long time at the evolutionary scale of time, which may even be millions of years (McCutcheon et al., 2009; Wernegreen, 2002).

One of the consequences of this long lasting coevolution, which could be considered as a potential negative effect, is the substantial gene loss and a severe genome shrinkage. Endosymbionts harbours the smallest genomes reported to date, although the symbiont gene sets retain the genes that are essential to the host. This phenomenon questions the nature of true essential genes (Moran and Bennett, 2014; McCutcheon and Moran, 2012).

Buchnera aphidicola is an example of primary endosymbiont as it synthesizes and provides essential amino acids for the different aphid species it infects (Chong et al., 2019). Another example of long lasting mandatory association between a primary symbiont and its host is the association between the bacterium *Sulcia muelleri* and its sharpshooter host, or other hosts from the Auchenorrhyncha suborder (Order: Hemiptera), for instance, *Macrosteles quadrilineatus* or *Homalodisca vitripennis* (Bennett and Moran, 2013; Moran et al., 2005b) lasting since over 260 million years.

Secondary symbionts

Secondary symbionts are facultative, the host is not dependent on it for its survival but can confer beneficial effects and increase its fitness. For these symbionts, although interesting, the vertical transmission is non mandatory and the symbiont rather switches between different host species, leading to occasional horizontal transfers, as indicated by the total lack of phylogenetic congruence and thus the recent symbiotic origin (Dale and Moran, 2006). Unlike primary symbionts, the genome size of secondary symbionts is not severely reduced, but ranges from the typical size of free-living bacteria to an intermediate reduction, depending on when the symbiont was associated with its host. This shows a transition in the genome reduction (Toh et al., 2006; Moran et al., 2005a; Wu et al., 2004;

Dale et al., 2006).

For example, the pea aphid *Acyrthosiphon pisum* infected by the bacterial endosymbiont *Hamiltonella defensa* is protected from parasitism by the wasp *Aphidius ervi* by killing the parasitoid larvae (Oliver et al., 2003; Oliver et al., 2009b; Oliver et al., 2009a). Many other examples of protection against natural enemies (pathogens, parasites, predators) occur in invertebrates (Haine, 2008). Another benefit provided by facultative symbionts is the expansion of the host ecological niche, for instance strains of the pea aphids *A. pisum* infected by *Regiella insecticola* symbiont enhance the feeding performance on a greater diversity of plants, suggesting that *R. insecticola* infections can increase the host fitness (Tsuchida et al., 2004).

Bacterial genus and expected phenotype

Another type of association exists with the reproductive parasite, with notably the famous example of *Wolbachia*. This genus is estimated to infect around 50% of insect species. However, its detection in a given arthropod population is not necessarily associated with a high prevalence (Sazama et al., 2019). This manipulator of reproduction uses several strategies — feminization, parthenogenesis, male killing and sperm–egg incompatibility — depending on the infected species to ensure its vertical transmission (Werren et al., 2008). Nonetheless, this genus is not limited to the reproductive manipulation behaviour, in certain cases *Wolbachia* are obligate mutualists in bedbugs (Hosokawa et al., 2010; Nikoh et al., 2014) and in filarial nematodes (Fenn and Blaxter, 2004). Those symbionts from the same genus display a wide range of diverse phenotypes (Zug and Hammerstein, 2015).

A parallel can be depicted with the *Rickettsia* genus, where most of the *Rickettsia* are often considered as obligate intracellular pathogens (Perlman et al., 2006), but cases of possibly mutualistic interactions with its host have been described, notably in ticks, with *R. peacockii* in *Dermacentor variabilis*, *R. buchneri* in *Ixodes scapularis* or *Rickettsia* sp. endosymbiont of *I. pacificus* (Felsheim et al., 2009; Kurtti et al., 2015; Hunter et al., 2015).

It is assumed that around half of arthropod species are infected with *Wolbachia* and around a quarter are infected with *Rickettsia*, showing the widespread Rickettsiales oc-

currence among Arthropoda (Weinert et al., 2015).

Symbionts of blood-feeding arthropods

The blood constitutes a very specialized diet for obligate blood-feeding arthropods, by containing disproportionaly high amounts of proteins, iron, salt, in comparison with lipids, carbohydrates, cofactors or vitamins. This imbalance has led to the evolution of gene sets capable of managing the nutrient inputs that may be toxic in excess as well as counteracting some of the nutritional deficiencies. However, this genomic dependence is not sufficient in the case of the biosynthesis of certain essential micronutrients that these animals are unable to produce. They had to rely on the presence of a functional microbiome with nutrient endosymbionts capable of supplying several essential cofactors and B vitamins (Duron and Gottlieb, 2020) . The wide variety of bacterial genera providing B vitamins among blood-sucking arthropods establishes a nutritional convergence (Duron and Gottlieb, 2020). Ticks are no exception to this dogma by mainly relying on diverse phylogenetically unrelated bacterial classes, such as Mollicutes, Sphingobacteriia, Gamma- and Alpha-proteobacteria (Bonnet et al., 2017; Fig. 2).

Ticks

Hematophagy evolved more than 20 times among the phylum Arthropoda (Mans, 2011), and is therefore occuring in several insect orders such as Diptera (e.g. mosquitoes, Tsetse flies), Anoplura (e.g. louse), Siphonaptera (e.g. fleas) or Hemiptera (e.g. triatomine, bed bugs) (Hocking, 1971). This behaviour is also common among the more than 55,000 described Acari species, comprising both mites and ticks (Zhang, 2013). Ticks, or the order Ixodida, are indeed exclusively hematophagous. The name Ixodida comes from ixôdês in ancient Greek, meaning 'sticky', probably referring to the fact that ticks are strongly 'stuck' to their host skin (and difficult to detach) thanks to their claws and pulvilli located at the extremity of their four pairs of legs. Ticks are even more tightly attached to their host once they have inserted their harpoon-like mouthparts (hypostome) in the skin of their host to take their blood meals. Around 900 species of ticks have been described all over the world, belonging to four families: (i) Deinocrotonidae (extinct family, with the

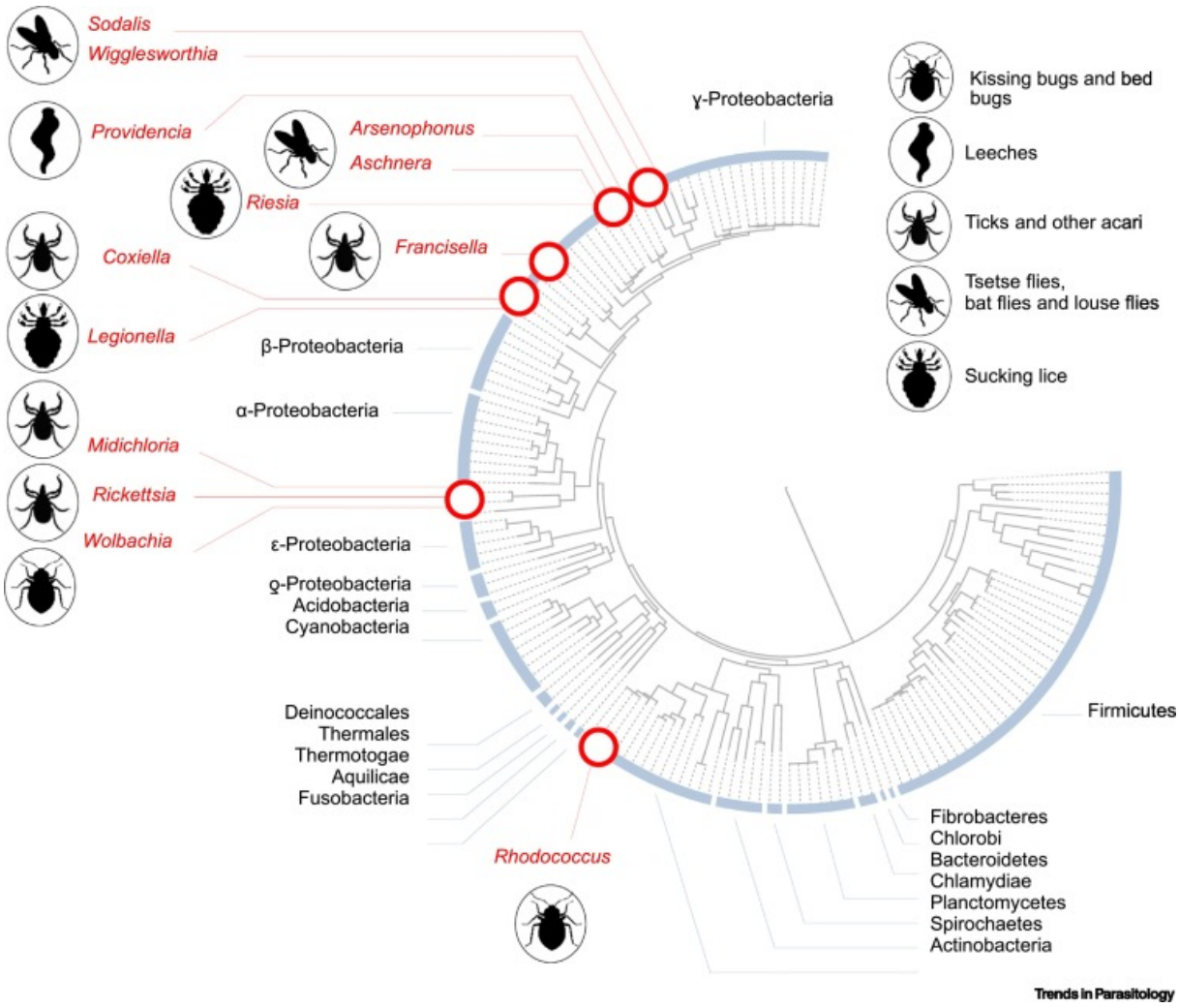

Figure 2 – **Evolutionary relationships of major examples of B vitamin-provisioning symbionts associated with obligate blood feeders.** The phylogeny is based on widely supported findings from studies listed in the citations. B vitamin-provisioning symbionts associated with OBFs are shown in red (from Duron and Gottlieb, 2020)

only species known *Deinocroton draculi*; Peñalver et al., 2017) , (ii) Nuttalliellidae, with also only one species known *Nuttalliella namaqua*, (iii) Argasidae (or soft ticks) with 208 species (Nava et al., 2017), and (iv) Ixodidae (or hard ticks) with ca. 700 species (722 according to Nava et al., 2017). Hard ticks are called as such for their hard, chitinous tegument, characteristic absent from the Argasidae. They are indeed characterised by the presence of a scutum, a dorsal sclerotized plate, directly posterior to the head. Within the Ixodidae, two groups are recognized, the Prostriata (or Ixodinae), corresponding to the sole *Ixodes* genus (250 species) and the Metastriata or Amblyomminae, including 12 genera such as *Amblyomma*, *Dermacentor*, *Hyalomma* or *Rhipicephalus*.

Ticks have been studied with great attention for decades, notably because of the transmission of tick-borne pathogens, potentially harmful to their vertebrate hosts. The list of known tick-borne pathogens is evolving and even growing, and includes a wide variety of bacteria, viruses and other parasites that have a definite impact on the health of humans, livestock and certainly wild animals (Fuente et al., 2008; Boulanger et al., 2019; Madison-Antenucci et al., 2020). New-born larvae are thought to be essentially free of pathogens, vertical transmission occurring rarely. Although, studies have recorded maternal transmission for each kind of disease agent (Chauvin et al., 2009; Burgdorfer et al., 1980; Xia et al., 2016). Also for instance, in *Ixodes scapularis*, *Borrelia burgdorferi* sensu stricto is considered not to be transmitted to the progeny, unlike *Borrelia miyamotoi* (Han et al., 2019). Thus, studying ticks, their feeding behaviour, life cycle and ecology are essential to understand and reduce health risk.

Feeding style and life cycle of hard ticks

All ticks have to feed on the blood sucked from a host to develop. Hard ticks have a three-stage life cycle, *i.e.* larva, nymph and adult (where male and female exhibit a strong sexual dimorphism), along with the egg stage. The moultings occur between the larva and nymph stages (where the larva with six legs acquires the fourth visible pair of legs; Santos et al., 2013), then between the nymph and adult stages. To develop to the next stage, a blood meal is required, leading to three obligate blood meals needed for the development of a hard tick from its hatching to the female egg-laying, which will give birth to a new generation of hundreds or thousands of larvae (Fig. 3).

Indeed, the ingested blood is metabolized in various nutrients needed for the development and the production of a new body or thousands of eggs. Contrary to mosquitoes,

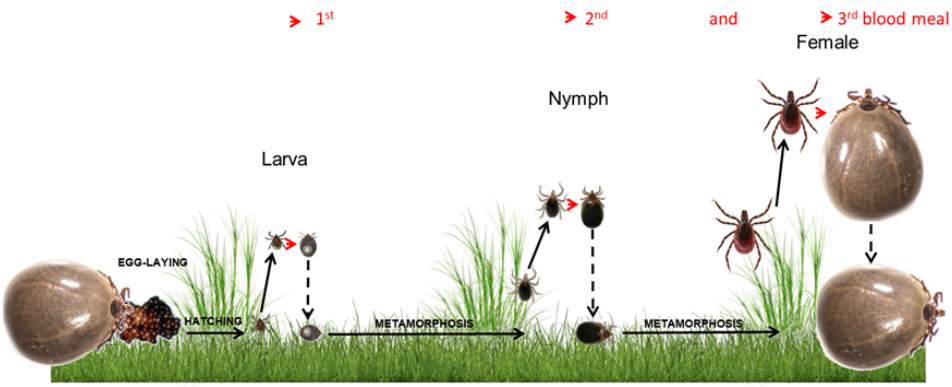

Figure 3 – **Life cycle of the hard tick *Ixodes ricinus***. Credit: A. Agoulon and O. Plantard

which spend a large part of their life cycle as aquatic eggs, larvae and pupae which feed on plankton, or even female adults that also use flower nectar as a source of energy, ticks are strictly hematophagous and do not ingest any other sources of nutrients than the ones provided by their blood meal. Adult females, but not adult males (with few exceptions), need a third blood meal, lasting several days to more than a week to furnish the nutrient uptake necessary to the development of thousands of eggs, which occurs also after mating. The adult female of *I. ricinus* can roughly uptake one millilitre of concentrated blood (Balashov, 1972). Indeed, the cycle cannot continue without this peculiar event, even for rare parthenogenetic species (Oliver, 1971; Oliver, 1981). One of the major adaptations of ticks is their ability to survive without feeding for months or even years, questing for a host for their blood meal. Some species perform their life cycle on a single host (Roberts, 1968), however like most of the Ixodidae, *I. ricinus* has a three-host cycle, each blood meal being followed by a detachment from the host and a fall on the ground where the moulting happens (Černý et al., 1974).

Ixodes ricinus ecology

The European tick *I. ricinus*, also known as 'castor bean tick' or 'sheep tick', is the most widespread tick in Europe (Estrada-Peña et al., 1998), from Scandinavia to Spain and from Portugal to Russia (Medlock et al., 2013. *Ixodes ricinus* is a generalist tick, meaning that its host specificity is very low (not only humans but mostly wildlife and livestock are

used as hosts), which is one of the keys its success as a disease vector (Socolovschi et al., 2009). That species is a three-host tick. Larvae of *I. ricinus* usually feed on small hosts such as micro-mammals (e.g. voles, mice, shrews), squirrels, birds and lizards, but can be found on virtually all vertebrates. Nymphs use preferentially medium to large mammals, while female adults are commonly found on roe deers or wild boars but almost never on micro-mammals (Pérez-Eid, 2007). Humans and cattle constitute accidental hosts. The time spent on-host is incredibly reduced compared to the off-host time. Due to its sensibility to dessication, *Ixodes ricinus* alternates between hiding on the ground, among the litter for instance to rehydrate, waiting for suitable environmental conditions, and host-seeking. The host-seeking, or questing strategy, consists classically to perch on a plant (e.g. rush, grass spike), which height is stage-dependent. Like many other ticks, *I. ricinus* has a complex of sensory organs (with pedipalps, sensory bristles and Haller's organ on the first pair of legs) that maximises the detection and perception of its future vertebrate host, through for instance olfaction, sensing of humidity, temperature, and carbon dioxide. Thus, a tick with the first pair of front legs extended is a sign that it is seeking and waiting for the passage of a host, where the tick will try to cling among the hairs. Therefore, its life cycle lasts in the wild up to two or three years with roughly one year between each host (Dantas-Torres and Otranto, 2013). However, it is possible to reduce the cycle duration under laboratory conditions but hardly less than in one year.

Symbionts of ticks

To date, ten bacterial genera are found to be commonly associated with ticks ensuring vertical transmission from the mother to the progeny (Duron et al., 2017; Bonnet et al., 2017; Bonnet and Pollet, 2020; Fig. 5).

The most common genera are *Coxiella*-Like Endosymbionts (ca. 50% tick species found to be infected) and *Francisella*-Like Endosymbionts (20%) (Duron et al., 2017). *Rickettsia* is also common, *Midichloria* is a bit rarer. The other remaining genera are *Spiroplasma*, *Arsenophonus*, *Cardinium*, *Rickettsiella*, *Wolbachia* and *Arsenophonus*. Among the possible roles of those symbionts, the most convincing hypothesis is the one related to nutritional symbiosis (Duron et al., 2018; Duron et al., 2017; Duron and Gottlieb, 2020; Gottlieb et al., 2015). Symbionts would provide B vitamins, notably riboflavin (B2), bi-

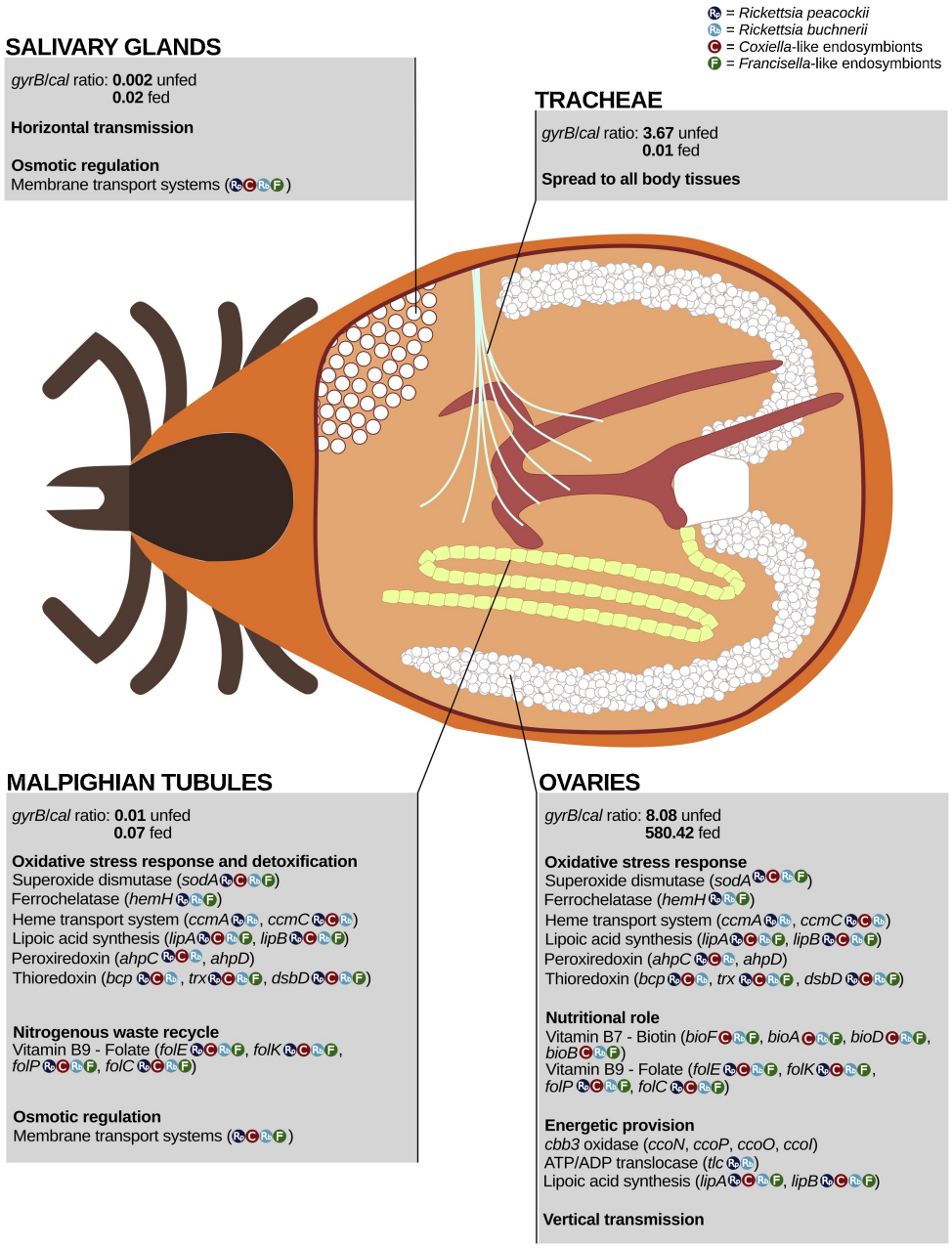

Figure 4 – **Schematic overview of the tissue localization of *M. mitochondrii* within its tick host.** Using comparative genomic data, the biological roles of *M. mitochondrii* and other tick symbionts are speculated using comparative genomic data. For each organ, the *gyrB/cal* ratios corresponding to the *M. mitochondrii* density in unfed and partially-fed females are displayed along with the symbiont inferred functions, the putative involved genes and whether homologs of the genes were found in other tick symbionts (from Olivieri et al., 2019).

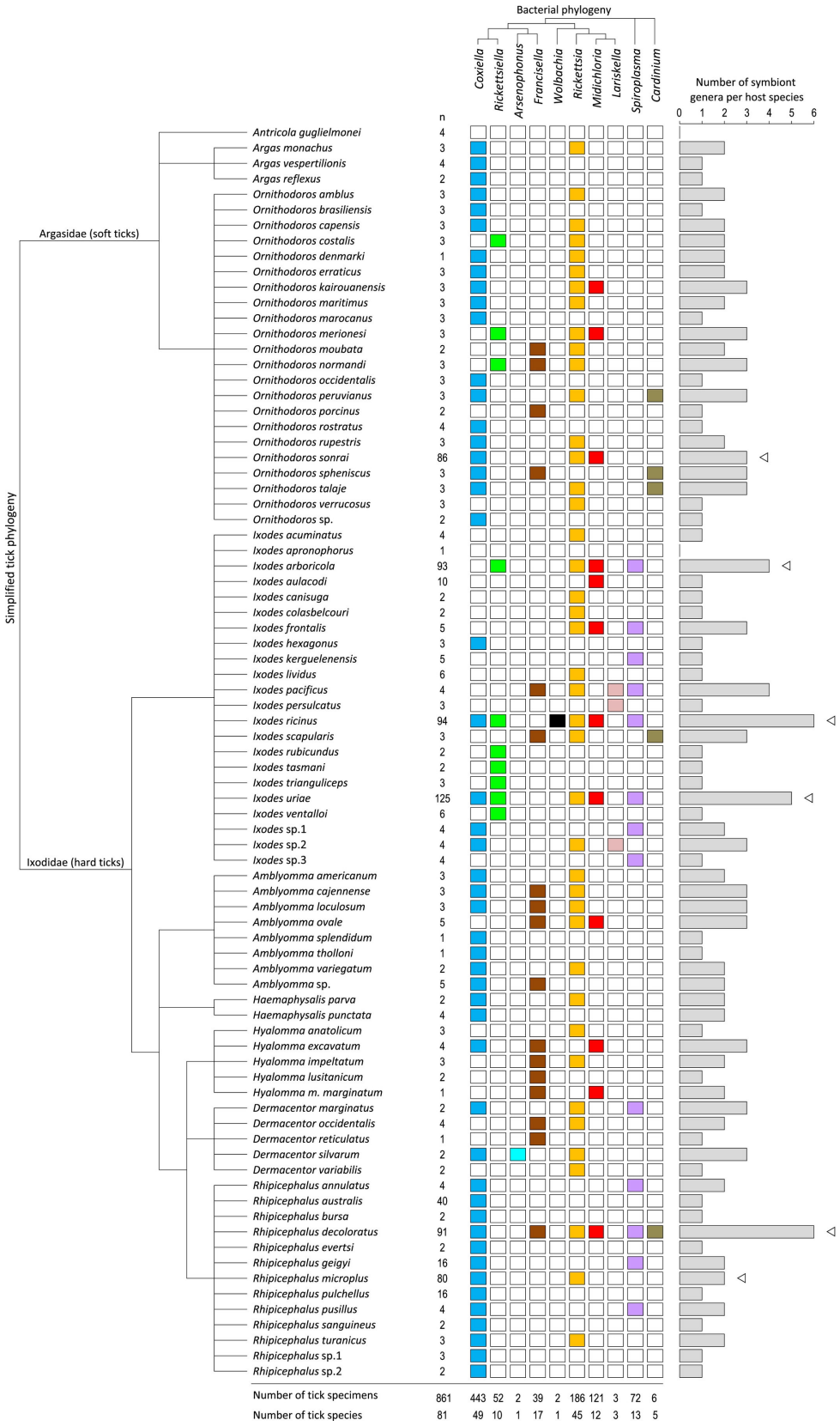

Figure 5 – **Distribution of 10 heritable bacterial symbionts within 81 tick species** (from Duron et al., 2017).

otin (B7), folate (B9), through pathways which are shared by *Coxiella*-like endosymbionts and *Francisella*-like endosymbionts. Thus ca. 70% of the symbionts encountered in ticks could provide those three vitamins.

The *Midichloria* genus has been firstly described in *I. ricinus* and the only species described to date, *M. mitochondrii*, appears to be the main symbiont of *I. ricinus* (Sassera et al., 2006).

Midichloria (Alphaproteobacteria: Rickettsiales: Midichloriaceae)

Rickettsiales

The Rickettsiales are an order of the Alphaproteobacteria class. The entire diversity of the Rickettsiales might have not been entirely discovered (Darby et al., 2007).

The Rickettsiales display various phenotypes within their association with their host, they can be for instance human pathogens (Kocan et al., 2004; Parola et al., 2013; Weinert et al., 2009), mutualists of nematodes (Taylor et al., 2005; Werren et al., 2008) or arthropod (Hosokawa et al., 2010), or even reproductive parasites of arthropods (Werren et al., 2008; Kang et al., 2014; Fig 6). Due to the lack of congruence between host and symbiont phylogenies, it can be inferred that one characteristic of most Rickettsiales is their faculty to perform horizontal transmission (e.g. Dantas-Torres et al., 2012; Epis et al., 2008; Kocan et al., 2004; Matsuura et al., 2012; Perlman et al., 2006; Weinert et al., 2009) but also odd host transfer (Braig et al., 1994; Caspi-Fluger et al., 2012; Schulz et al., 2016; Senra et al., 2016). All Rickettsiales bacteria are known to be obligate intracellular Alphaproteobacteria (Salje, 2021), with one exception of a Rickettsiales bacterium associated with *Paramecium* (Castelli et al., 2019).

Midichloria mitochondrii

Midichloria is a genus of the Alphaproteobacteria class, the Rickettsiales order and the Midichloriaceae family (Sassera et al., 2006; Montagna et al., 2013), and was found to be a candidate symbiont of several tick species (Epis et al., 2008; Cafiso et al., 2016; Duron

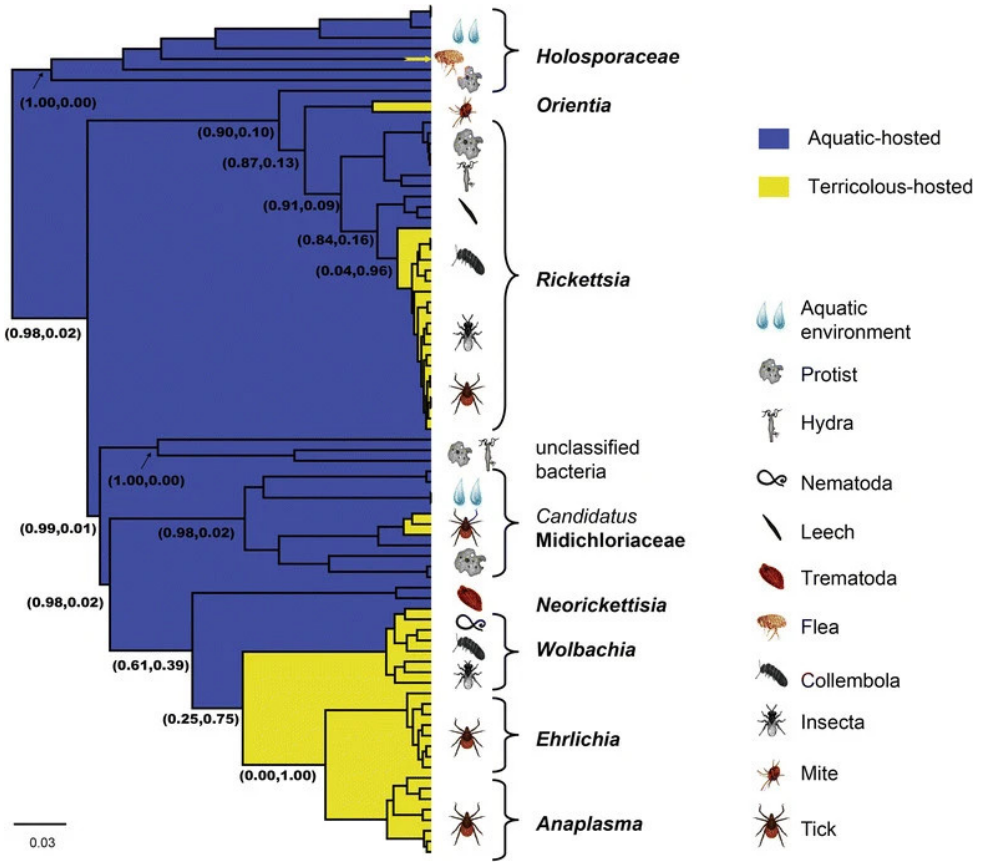

Figure 6 – **Molecular phylogeny of the order Rickettsiales based on partial 16S rRNA gene sequences** (from Kang et al., 2014).

et al., 2017).

As key organs for the reproduction and the production of the progeny, ovaries are also the main organ where *M. mitochondrii* is found at high density (Guizzo et al., 2020; Olivieri et al., 2019; Sacchi et al., 2004; Sassera et al., 2006; Zhu et al., 1992). Historically, they were the very first organ where *Midichloria* have been observed as it was initially identified (Lewis, 1979) "within the ooplasm and the mitochondria of developing oocytes". It has been subsequently found in ovarian primordia of *I. ricinus* immatures (Zhu et al., 1992) and described as *Rickettsia*-like microorganisms based on bacterial morphology investigated through electron microscopy.

Then, *Midichloria* has been formally described in 2006 (Lo et al., 2006; Sassera et al., 2006); *Midichloria* is coined after the Star Wars movie saga, referring to microscopic intelligent life forms, Midi-chlorians; *mitochondrii* stands for the bacterium ability to reside within mitochondria, between the inner and outer membrane (Beninati et al., 2004; Sacchi et al., 2004). To my best knowledge, it is still the only bacterium able to reside within the mitochondria among the Metazoa kingdom (Fig. 7).

Besides being the only bacterium described in the genus *Midichloria*, this bacterium has initiated the creation of the Midichloriaceae family, a new family within the Rickettsiales order, where intracellular bacteria exhibiting diverse phenotypic trait of their host (that inhabits various environments) have been identified (Montagna et al., 2013).

There is no evidence of co-cladogenesis for *Midichloria* within ticks (Buysse and Duron, 2018) and even within *I. ricinus*, except for Scottish populations (Al-Khafaji et al., 2019).

The sequencing of *M. mitochondrii* genome did not reveal any genome reduction (spanning around 1.2Mb), with no evident essential gene or function loss (Sassera et al., 2011). These arguments are in favour of a recent acquisition of *M. mitochondrii* at the evolutionary scale of time by *I. ricinus*. The bacterium has the genomic ability to synthesise two B vitamins and other essential cofactors (Sassera et al., 2011) that ticks are not able to synthesise (Gulia-Nuss et al., 2016; Jia et al., 2020 or to gain through the blood meal (Duarte et al., 1999). However, the bacteria seem to be fixed in *I. ricinus* populations (Guizzo et al., 2020; Lo et al., 2006), at least in the wild. Those points could indicate a

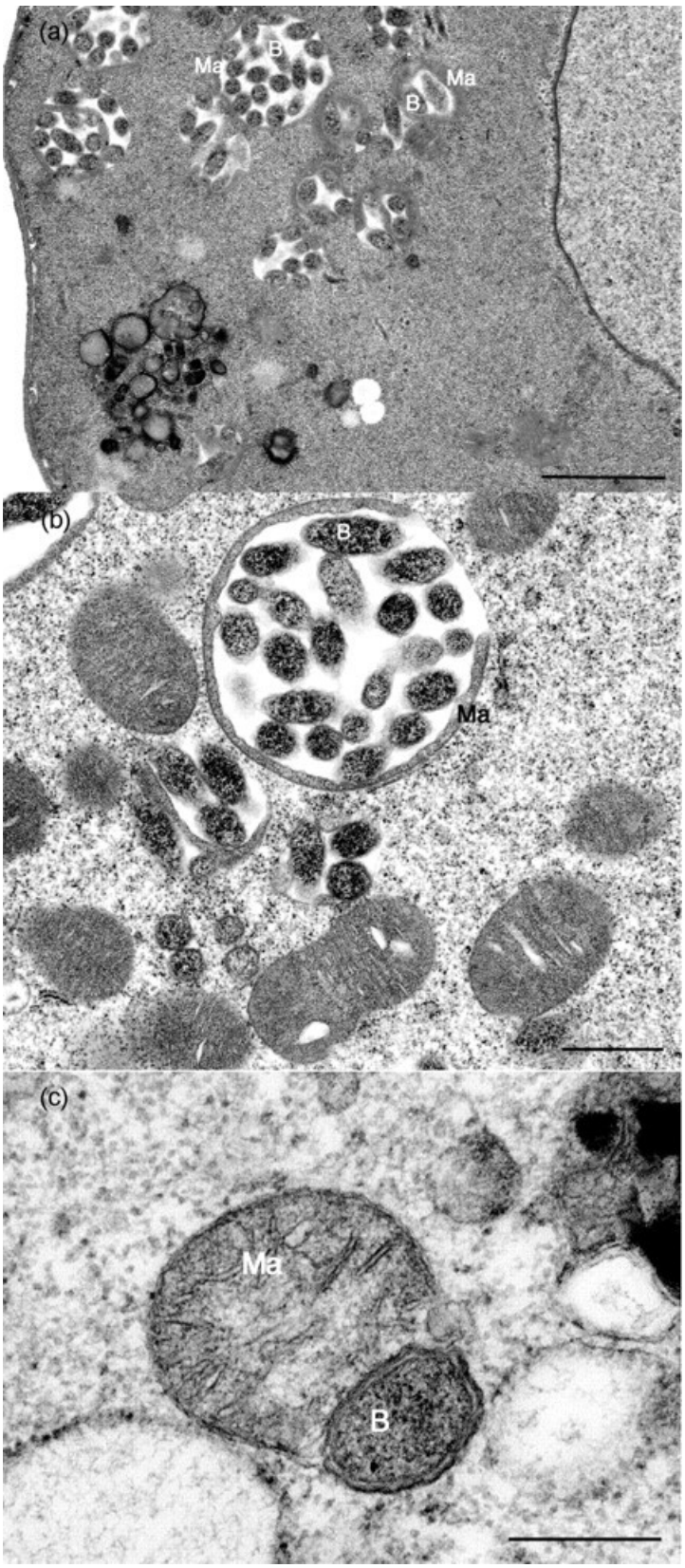

Figure 7 – **Electron micrographs of ovarian cells of *I. ricinus* containing 'Candidatus *M. mitochondrii*** (B) within intact or degraded mitochondrial matrix (Ma). Bars, 2.4µm (a), 1.2µm (b) and 0.45µm (c) (From Sassera et al., 2006)

recent obligate mutualism between *I. ricinus* and *M. mitochondrii*. However, the apparent loss of *M. mitochondrii* in the laboratory strains may indicate that the symbiont is only essential under wild conditions (Lo et al., 2006), which also questions a potential facultative trait. Furthermore, the bacteria are supposed to be essential only for females as *M. mitochondrii* is undetectable in 56% of males (Lo et al., 2006) at a very low density (Sassera et al., 2008).
Bacteria related to *M. mitochondrii* have since been found on numerous occasions in ticks. It appears that the prevalence of *Midichloria* in those tick species is much more often intermediate (or even rare) than ubiquitous (Cafiso et al., 2016; Duron et al., 2017; Epis et al., 2008). This suggests a different phenotype and interplay with a tick host different than *I. ricinus*.
In addition, while the intramitochondrial tropism of *M. mitochondrii* could be considered parasitic, *I. ricinus* females do not have apparent problem laying eggs and producing viable larvae (Sassera et al., 2006). However, the question of how long the interaction between the symbiont and its host has lasted on evolutionary time scales may be raised.

Key organs involved in the interplay between *Midichloria mitochondrii* and *Ixodes ricinus*

I focused during my thesis on the ovary, the Malpighian tubules and the salivary glands because these organs have already been reported in the litterature as harbouring *Candidatus* Midichloria mitochondrii (hereafter *Midichloria mitochondrii*) (Sassera et al., 2006; Olivieri et al., 2019; Fig 4). Of note, in this article the finding of *M. mitochondrii* in tracheae would rather be related to the presence of tick fat bodies (that are especially difficult to distinguish as they are constituted by few cells or ribbons of cells; Denardi et al., 2008) stuck to tracheae. Other possible relevant organs — midgut and hemolymph — where *M. mitochondrii* could be found have not been investigated in the present work due to technical issues, notably the presence of PCR inhibitors and sampling difficulties respectively. This section briefly presents some characteristics of these organs.

The ovary

In females, the ovary (an unpaired organ in hard ticks, contrary to testes) is a simple tube-like structure in the juvenile stages and in the unfed female (Sonenshine and Roe,

2013), that inflates upon engorgement and mating to receive developed oocytes (Fig 8, 9 and 10). Ovaries are the most important interface between *M. mitochondrii* and the tick mainly because they allow vertical transmission of the symbiont, thus allowing *M. mitochondrii* to infect the progeny. Within the ovary tissue, *M. mitochondrii* has been found within oocytes and in the funicle cells.

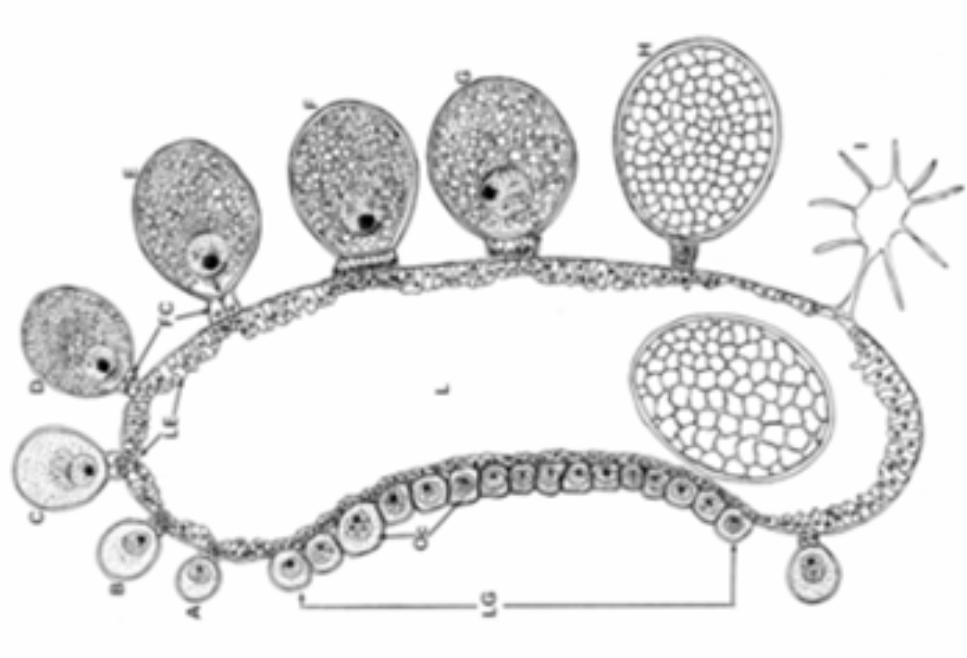

Figure 8 – **Various stages of oocyte development and maturation can be observed along a section of the same part of a tick ovary.** (from Sonenshine and Roe, 2013).

Malpighian tubules

Malpighian tubules are also an organ where *M. mitochondrii* have been found but at much lesser extent and density than ovary (Olivieri et al., 2019). Malpighian tubules are thought to have a similar function of kidney-like organs, found also in other arthropods, where they are believed to be in charge of detoxification, osmoregulation and excretion of metabolic wastes, especially to get rid of nitrogenous ones (Sonenshine and Roe, 2013). In ticks, there is only a single pair of those organs at the contrary of many insects and they

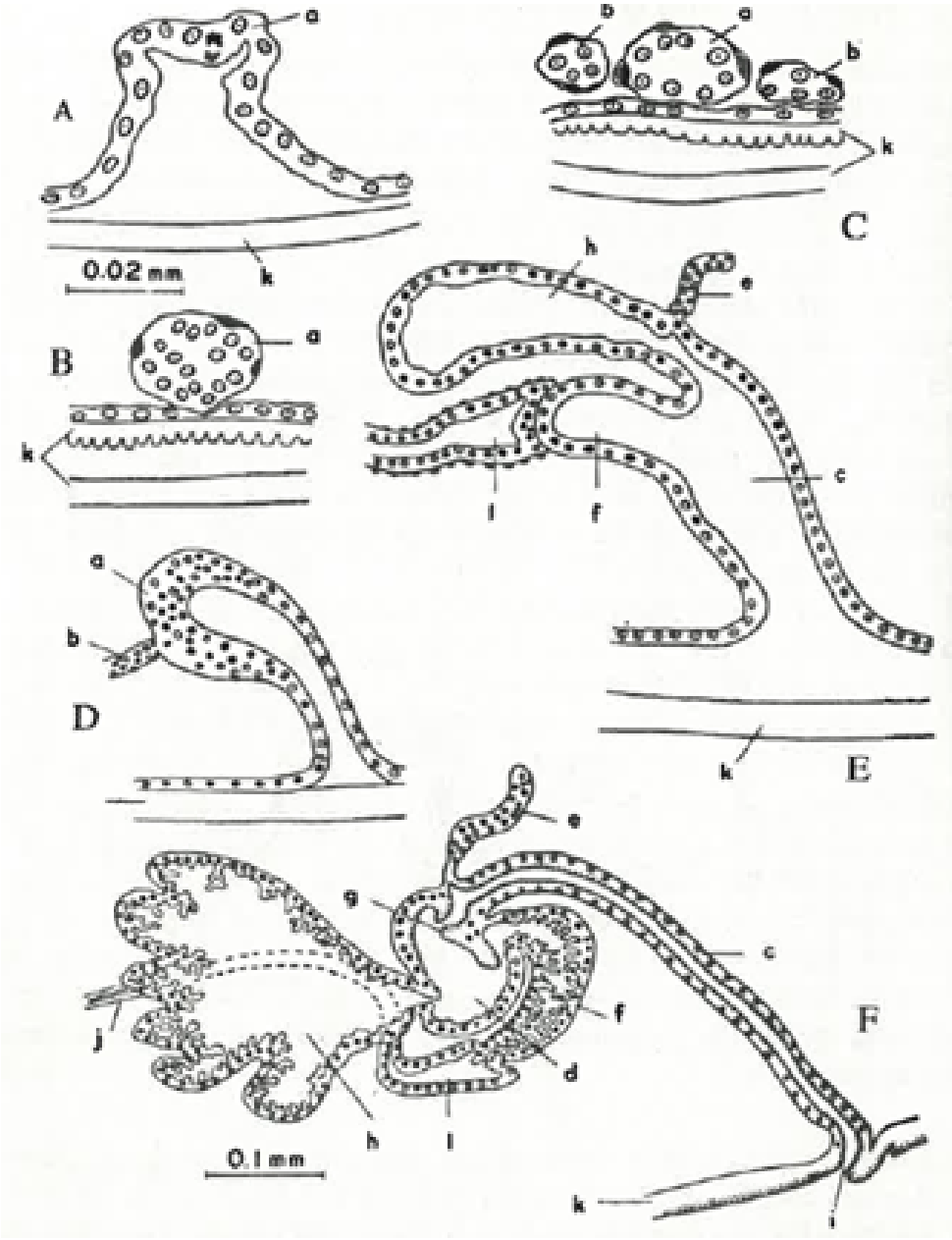

Figure 9 – **Illustration of female gonad development during ontogenesis (in larvae, nymphs and adult females).** Development of the genital apparatus of the female *Hyalomma asiaticum* (sagittal sections). A. Ungorged larva at the beginning of metamorphosis. B. Larva at mid-metamorphosis. C. Larva at the end of metamorphosis. D. Unfed nymph. E. Nymph in metamorphosis. F. unfed female. a:vagina and primary accessory glands. b:primary oviduct. c and d: vestibule and cervical region of vagina respectively. e: tubular accessory gland. f: connective tube. g: valve. h: seminal receptacle. i: external genital opening. j: oviduct. k: cuticle. l: odd oviduct (from Pérez-Eid, 2007).

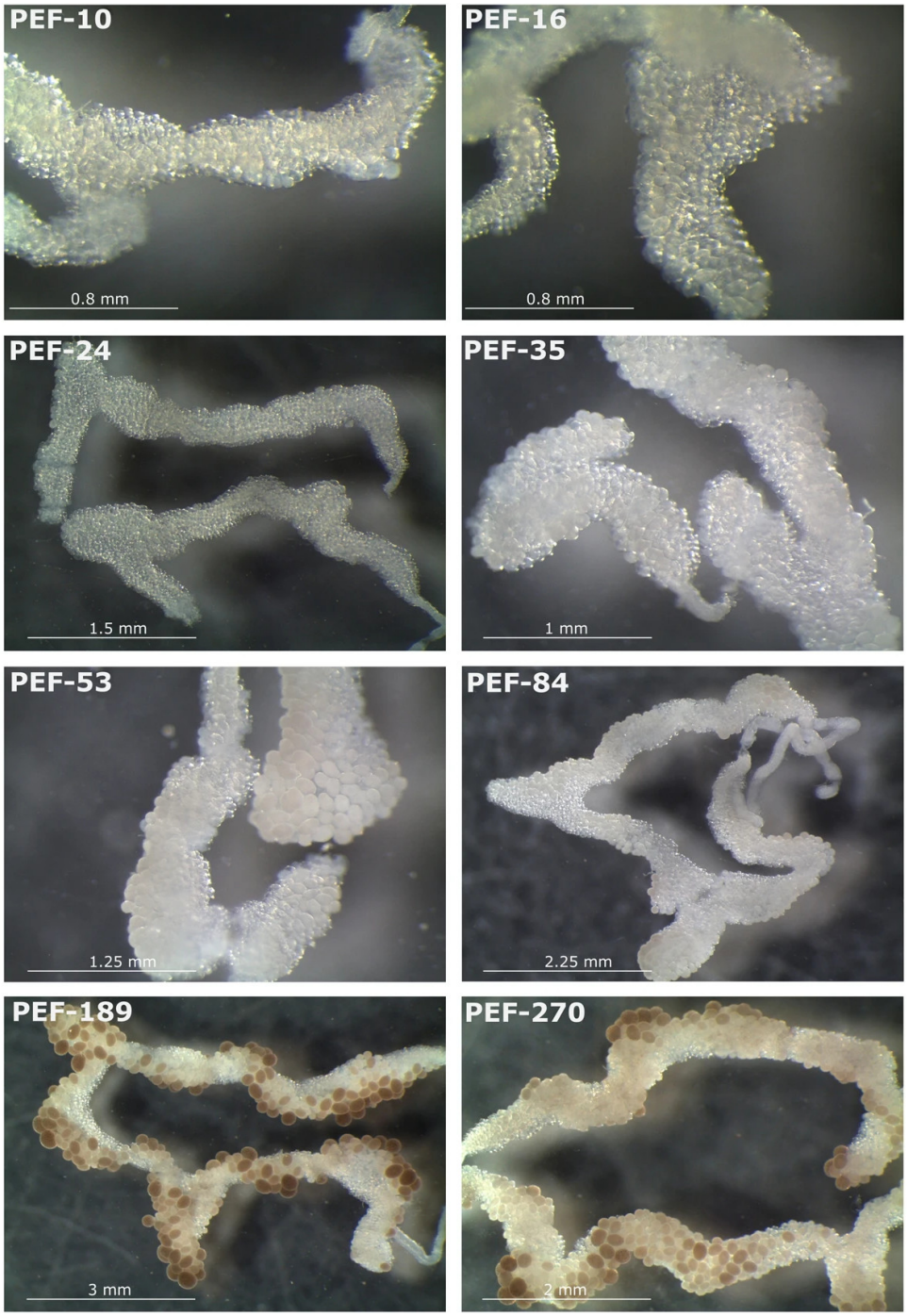

Figure 10 – **Representative images of specimens and ovaries of Rhipicephalus microplus partially engorged females (PEF) groups.** PEF were grouped according to average weight of individuals (10±1.73mg, 16±1.22mg, 24±1.90mg, 35±2.84mg, 53±2.49mg, 84±8.36mg, 189±17.17mg, 270±16.71mg). Ovary images (from PEF-10 to PEF-270) exemplify the ovaries from PEF groups used in this study, which correspond to ovarian growth phase (OGP) (from Xavier et al., 2018).

are connected to the rectal sac (distal part of the midgut) near the anal pore. Those wastes are in the form crystals of guanine (2-amino-6-oxypurine) (Hamdy and Sidrak, 1982) and they transit from the hemolymph through the Malpighian tubules to finally the rectal sac from where they are excreted. Malpighian tubules have also been hypothesized to be involved in water elimination (Ball et al., 2009). At the adult unfed stage, these organs can be easily mistaken with the trachea at first sight, even though the texture and structure is slightly different. However, after the beginning of the engorgement, they start to inflate and develop and are easily distinguishable from other organs. Three distinct regions of the Malpighian tubules have been described (distal, main and proximal).

Salivary glands

Salivary glands are perhaps the most well documented organs in tick literature because of their importance for pathogen colonization and transmission (Šimo et al., 2017, Fig. 11).

Its importance in tick physiology is due to the release of compounds into the host blood targeting mechanisms such as angiogenesis (wound healing), platelet aggregation, blood coagulation, innate and acquired immune response (reviewed in Šimo et al., 2017). They are also key organs to maintain water balance. In female hard ticks, grape-like salivary glands clusters are composed of three types of spherical acini (type I, II, and III, Fig. 12).

Following the blood meal, the volume of the salivary glands expands dramatically, highlighting their enhanced metabolism. *Midichloria mitochondrii* have been found in *I. ricinus* salivary glands and could thus be transmitted to the blood of vertebrates following blood meal, as experimentally demonstrated in Cafiso et al., 2018). *Rickettsia buchneri*, the main endosymbiont associated with *I. scapularis*, has also been demonstrated to colonise the salivary gland of its host too (Al-Khafaji et al., 2020). The main symbiont of *Amblyomma americanum* (*Coxiella*-like endosymbiont) has also been observed in the salivary glands of their tick-host suggesting that the presence of endosymbionts that are mostly concentrated in tick ovaries but are also directly connected to the blood system of vertebrates during the blood meal (Klyachko et al., 2007).

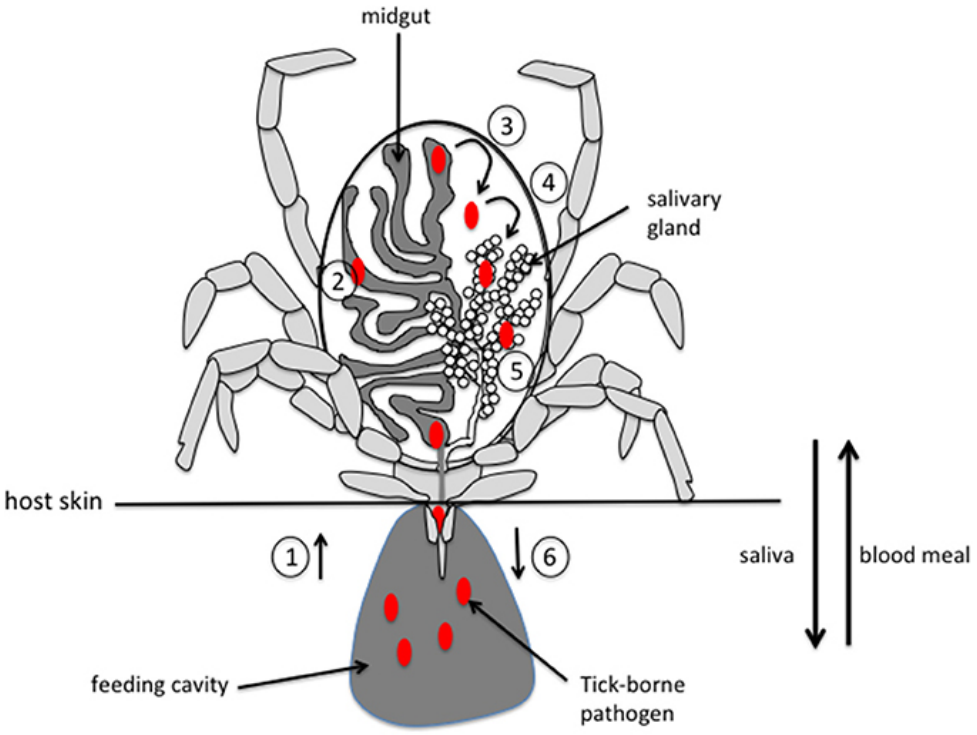

Figure 11 – **Schematic representation of pathogen acquisition, development and transmission by a tick** (1) pathogens are ingested by the tick along with the blood meal during the bite. (2) Pathogens invade the midgut and, depending on the species, stay in the midgut until the next feeding or immediately cross the epithelium of the digestive tract (3) to invade the tick body. (4) Pathogens move into the salivary glands by crossing the epithelium and invade the acini (5). (6) Pathogens are injected into a new host during feeding, along with saliva that counteracts host hemostasis, inflammation and immune responses, thus facilitating pathogen infection of host. Please note that, for clarity, only half of the digestive tract and a single salivary gland are represented. (From Šimo et al., 2017).

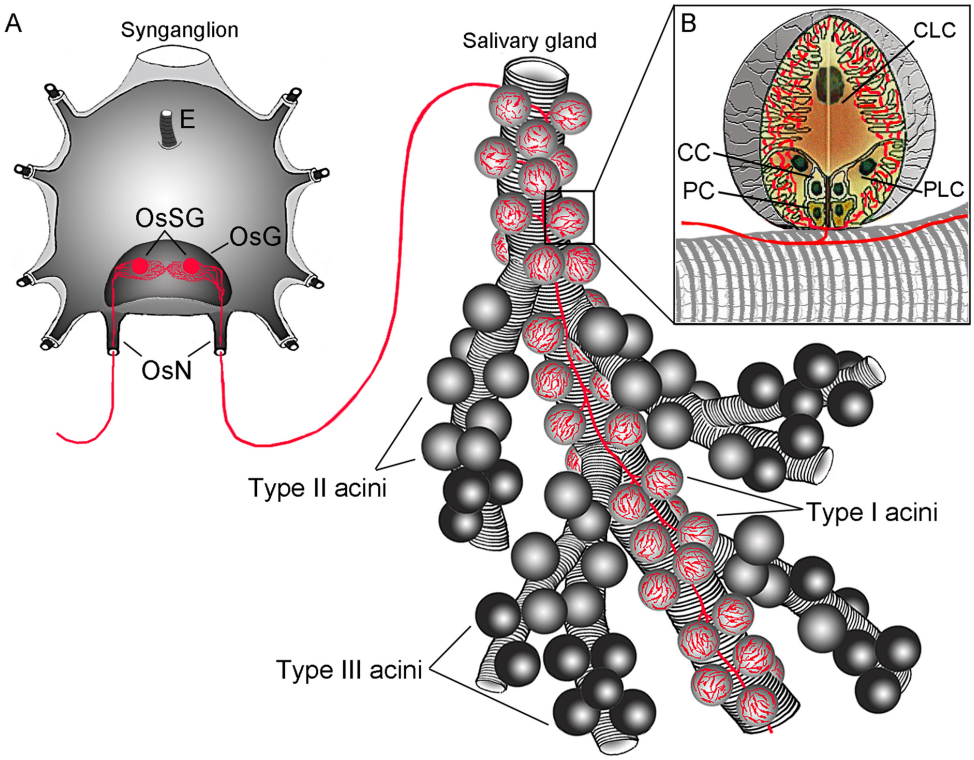

Figure 12 – **Schematic illustration of the cholinergic connection between the Ixodes synganglion and salivary gland.** (A) The cholinergic axons (red) originating from OsSG neurons exit the synganglion via the opistosomal nerves (OsN) to reach the type I acini exclusively. (B) Schematic detailing the distribution of cholinergic axons (red) within the type I acinus. Note that cholinergic axon terminals within the acinus invaginate between the basolateral infoldings of both peripheral lamellate cells (PLC) and the central lamellate cell (CLC). C circumlumenal cell, PC peritubular cell, E esophagus, OsG opistosomal ganglion (from Mateos-Hernandéz et al., 2020).

Thesis objectives

This thesis is divided into three main chapters which aim to answer different questions concerning the interplays between *M. mitochondrii* and *I. ricinus*. Two appendix chapters, dealing with investigations conducted on data sets or biological material obtained during this thesis, complete it.

- We hypothesize that female nymphs contain more bacteria compared to male nymphs, anticipating the difference in prevalence among sexes at the adult stage. Thus, to study the dynamics of *M. mitochondrii* in this sexually immature stage of *I. ricinus*, an experimental design was devised to infer the sex ratio of groups of nymphs (sex determination not being possible in the absence of sexual dimorphism at this stage and genetic marker) whose *M. mitochondrii* density was systematically studied.

- The attempts to create a symbiont-free tick line and a closely related control harbouring the symbiont allows to conduct comparisons of tick life-history traits in order to characterise the impact of the presence or absence of the symbiont on the host fitness. Those experiments were conducted by injecting antibiotics into unfed females. The density of symbionts in the offspring was then characterised. As with other endosymbionts providing B vitamins, the hypothesis of the vitamin supply by *M. mitochondrii* to *I. ricinus* was also tested in parallel.

- The impact of *M. mitochondrii* presence on tick gene expression was investigated to better understand the effect of the endosymbiont on its host . Therefore, for the first time to our knowledge, the transcriptomes of *I. ricinus* with and without the symbiont were compared, within several organs. Differentially expressed genes of ticks, in presence or absence of the symbiont, and thus potentially involved in the relationship with the symbiont, were characterised.

Chapter 1

Symbiont dynamics during the blood meal of *Ixodes ricinus* nymphs differ according to their sex

The following study has been published in the journal *Ticks and Tick-Borne Diseases* (TTBD).

Reference

Daveu, R., Laurence, C., Bouju-Albert, A., Sassera, D., Plantard, O., 2021. **Symbiont dynamics during the blood meal of *Ixodes ricinus* nymphs differ according to their sex**. *Ticks Tick-Borne Dis.* 12, 101707. https://doi.org/10.1016/j.ttbdis.2021.101707

1.1 Foreword

This work originates with Cindy Laurence's internship (2nd author, 2nd year Master student, supervised by Olivier Plantard in 2014), helped by Agnès Bouju-Albert (technician) between January and June 2014, aiming to describe the dynamics of *Midichloria mitochondrii* load in *Ixodes ricinus* nymphs following their engorgement. I re-analysed the whole data and conducted extensive statistical analysis to finally write a manuscript describing the results obtained. They provide new insights about *M. mitochondrii* load during a sexually immature stage of *I. ricinus* (nymphal stage), before its important multiplication in mature female gonads following blood meal (Sassera et al., 2008). That knowledge is hence useful to better appreciate the role of *M. mitochondrii* on the biology of its arthropod host.

1.1.1 Context

After having designed a qPCR protocol allowing the quantification of *M. mitochondrii*, Sassera et al., 2008 studied the dynamics of *M. mitochondrii* load across all tick life stages, from egg to adults, including the two intermediate stages (larvae and nymphs) both unfed and fed. In addition to the absolute number of symbiotic bacteria between adult females and males that was highly different, one noticeable result was the extensive variance of symbiont density observed in fed nymphs. This last observation was already interpreted as suggesting "a specialisation of the symbiont toward females at the nymphal stage". However, the interpretation of this high variance of symbiont density among nymphs was limited by the fact that the authors "were not able to determine the sex of (larvae and) nymphs". Thus, a study was specifically designed to evaluate with more accuracy and subtlety the symbiont density in a larger set of nymphs by comparing groups exhibiting different sex-ratios (given that a genetic marker of sex in *I. ricinus* is still not available to date).

1.1.2 Methods

Two hundred and fifty wild unfed *I. ricinus* nymphs were collected in January 2014 in Chizé forest, France. Three groups were built based on morphometric features (hypostome and scutum length), with the assumption that (i) the first group with the smallest measurement were mostly constituted of males, (ii) the second group with the intermediate hypostome and scutum lengths displays a 1:1 ratio male:female, (iii) the third group with the largest morphometric features were mostly constituted of females. Nymphs were weighted before engorgement, a part was sacrificed for Midichloria density investigation while the rest was submitted to a blood meal on a vertebrate host. After feeding, fed nymphs were weighed once more, a part was left moulting, while the rest was again sacrificed for Midichloria density investigation by qPCR.

1.1.3 Main results

While no significant difference in either body mass or *M. mitochondrii* load was observed at the unfed stage, both features were significantly different between each size group following engorgement. Our results demonstrate that symbiont dynamics during nymphal engorgement is different between the two sexes, resulting in a significantly higher *M. mitochondrii* load in nymphs that will become females. It suggests that the increase in

the size of the ovary primordium in nymphs that will become females following blood meal is a key process to explain the pronounced *M. mitochondrii* high multiplication during this part of the tick lifecycle.

Although there is an increasing number of articles surveying the dynamics of symbionts in females of various tick species (Oliver et al., 2020; Wang et al., 2018) following blood meal, this is, to our knowledge, the only study investigating symbiont dynamics in nymphs with such accuracy.

The development of ongoing genomic investigations and increasing amount of genomic resources (Murgia et al., 2019; Gulia-Nuss et al., 2016; Jia et al., 2020) should soon provide markers of the Y chromosome and thus allow to investigate the Midichloria load in individual nymphs of known sex.

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Symbiont dynamics during the blood meal of *Ixodes ricinus* nymphs differ according to their sex

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ABSTRACT

Ticks harbour rich and diverse microbiota and, among the microorganisms associated with them, endosymbionts are the subject of a growing interest due to their crucial role in the biology of their arthropod host. *Midichloria mitochondrii* is the main endosymbiont of the European tick *Ixodes ricinus* and is found in abundance in all *I. ricinus* females, while at a much lower density in males, where it is even absent in 56 % of the individuals. This endosymbiont is also known to increase in numbers after the blood meal of larvae, nymphs or females. Because of this difference in the prevalence of *M. mitochondrii* between the two sexes, surveying the density of these bacteria in nymphs that will become either females or males could help to understand the behaviour of *Midichloria* in its arthropod host. To this aim, we have set up an experimental design by building 3 groups of unfed nymphs based on their scutum and hypostome lengths. After engorgement, weighing and moulting of a subset of the nymphs, a significant difference in sex-ratio among the 3 groups was observed. In parallel, *Midichloria* load in individual nymphs was quantified by qPCR both before and after engorgement.

No difference in either body mass or *Midichloria* load was observed at the unfed stage, but following engorgement, both features were significantly different between each size group.

Our results demonstrate that symbiont dynamics during nymphal engorgement is different between the two sexes, resulting in a significantly higher *Midichloria* load in nymphs that will become females. The consequences of those findings on our understanding of the interplay between the endosymbiont and its arthropod host are discussed.

1. Introduction

Ticks are obligate hematophagous ectoparasites, vectors of numerous pathogens for both humans and animals (Jongejan and Uilenberg, 2004; Parola and Raoult, 2001). Beside pathogens, ticks harbour a diverse microbiome including commensal and symbiotic microorganisms (Bonnet et al., 2017; Duron et al., 2017). This is a common situation in arthropods, as most species are the host of various bacterial symbionts that can be transmitted transovarially to the progeny (Bennett and Moran, 2015; Wernegreen, 2012). During their long-lasting co-evolution with their arthropod hosts, symbionts have developed strategies to persist and multiply within their host. Some are obligate mutualists, essential for host fitness, whereas others may be facultative, allowing for example a better survival in adverse environmental conditions, providing resistance to natural enemies or manipulating host reproduction (Bennett and Moran, 2015; Cordaux et al., 2011; Engelstädter and Hurst, 2009; Haine, 2008; Oliver et al., 2003; Vorburger et al., 2010).

At least ten genera of symbionts have been reported in ticks, three of them being exclusive to ticks, *i.e. Coxiella*-like endosymbionts, *Francisella*-like endosymbionts and *Midichloria* (Bonnet et al., 2017; Díaz-Sánchez et al., 2019; Duron et al., 2017; Narasimhan and Fikrig, 2015). The obligatory role of some symbionts on tick fitness has been shown by removal through antibiotics (Ben-Yosef et al., 2020; Duron et al., 2018; Guizzo et al., 2017; Zhang et al., 2017; Zhong et al., 2007). In addition, a role as vitamin B provider has been demonstrated in the case of *Francisella*-like endosymbionts of *Ornithodoros moubata* (Duron et al., 2018).

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Candidatus Midichloria mitochondrii (hereafter *M. mitochondrii*) (Alphaproteobacteria: Rickettsiales: Midichloriaceae) is the main and most abundant symbiont of *I. ricinus,* the most common tick species in Europe (Guizzo et al., 2020; Sassera et al., 2006). Like all hard-tick species, *I. ricinus* displays a four-stage life cycle (larva, nymph, adult and egg). Each moulting (*i.e.* from larva to nymph and from nymph to adult) occurs after a blood meal, whereas egg-laying is conditioned by both female fertilisation and a third and final blood meal. *Midichloria mitochondrii* resides principally in oocyte cells (Beninati et al., 2004; Sassera et al., 2006; Zhu et al., 1992) but also, at a much smaller density, in salivary glands, Malpighian tubules and tracheae (Olivieri et al., 2019). Its density is also highly variable according to life stage and engorgement status, with a considerable bacterial growth observed following a blood meal in larvae, nymphs and females (Sassera et al., 2008). The bacterium exhibits a unique feature among symbionts as it resides within the host's mitochondrial intermembrane (Beninati et al., 2004; Stavru et al., 2020). This alphaproteobacterium is ~100 % prevalent in females and immatures (Aivelo et al., 2019), while a medium prevalence in males (~40 %) has been observed (Lo et al., 2006; Sassera et al., 2006). However, despite the high prevalence of these endosymbionts in *I. ricinus*, the consequences of the presence of *M. mitochondrii* in its arthropod hosts remain to be established experimentally.

Considering that (i) in *I. ricinus* there is a marked difference in *M. mitochondrii* prevalence between sexes, (ii) vertical transmission of *M. mitochondrii* has been demonstrated as it has been found in egg masses (Lo et al., 2006; Sassera et al., 2008), and (iii) an extensive variability of *M. mitochondrii* density between nymph individuals has been reported, especially in fed nymphs (Sassera et al., 2008), we designed the present study to investigate the dynamics of *M. mitochondrii* in this sexually immature stage of *I. ricinus*.

Such information could help us understand the interplay between the host tick and its symbiont *M. mitochondrii*. A first hypothesis could be that *M. mitochondrii* presence or density is determined by the genetic sex of ticks (in *I. ricinus*: XY for males, XX for females; Oliver, 1977) with a lower density or absence of *M. mitochondrii* in immature stages with a Y chromosome (*i.e.* nymphs that will become males). An additional hypothesis - not exclusive to the first one - could be that symbiont cells are restricted to the ovary and absent in the testes (the two organs being at a primordial stage in unfed nymphs but reaching a larger size following the blood meal and the metamorphosis process (Balashov, 1972). The number of endosymbionts may thus increase according to ovary development during ontogenic processes (while it may not increase following testes development). Note that tissue/organ location of *M. mitochondrii* has been investigated to date only in adults, due to the smaller size of immature stages such as nymphs (but see Epis et al., 2013). As *I. ricinus* do not show any sexual dimorphism at the nymphal stage while unfed, nymphs that will become either females or males cannot be identified prior to engorgement. Moreover, to date, no sexual genetic marker exists for ticks, despite ongoing investigations for whole genome sequencing of several *Ixodes* species (Gulia-Nuss et al., 2016; Jia et al., 2020; Murgia et al., 2019). To investigate the dynamics of *M. mitochondrii* in nymphs that will become either males or females, we set up an experimental design to examine groups of nymphs based on morphometric characteristics enabling to obtain different sex ratios in those groups. We then investigated *Midichloria* load in the nymphs of these groups, both before and after engorgement.

Finally, we will discuss the consequences of our findings concerning the evolution of *Midichloria* load during nymph development on our understanding of *Midichloria* biology and its interplay with its arthropod host.

2. Materials and methods

2.1. Study design

To discriminate between unfed nymphs that will become males *versus* females, we took advantage of a known size dimorphism (using body mass as a proxy) observed in nymphs fed at repletion that exhibit a marked bimodal distribution, with the heavier ones becoming females, and the lighter becoming males (Dusbábek, 1996; Kahl et al., 1990). This difference in body mass or morphometric features (such as idiosoma, scutum or hypostome lengths) between fed nymphs that will become males *versus* females is also present in unfed nymphs, but an extensive overlap is observed between the two distributions (that are not bimodal; Dusbabek 1996) preventing sex determination of individual nymphs prior to engorgement. In the present study, we built size groups based on morphometric features (*i.e.* hypostome and scutum lengths), with a hypothesised different sex-ratio, then we proceeded to the engorgement of the different groups separately, collecting a portion of unengorged and engorged nymphs prior to their moult to assess their *M. mitochondrii* load by qPCR (Fig. 1).

2.2. Ixodes ricinus ticks and morphological measurements

250 wild unfed *I. ricinus* nymphs were collected in January 2014 using the dragging method in Chizé forest, France (46°08′31.5″N, 0°25′22.5″W). Ticks were maintained in desiccators at 20 °C, 90 % relative humidity (with a saturated magnesium sulfate solution), 12 h dark:12 h light, until further use. Using a stereo microscope (Nikon SMZ800), a picture of each individual nymph was taken (Fig. 2). With the help of a 1 mm micrometric blade allowing to provide the scale of each picture, each nymph was characterized for the length of its scutum (rigid, sclerotised plate on the anterior dorsal surface, just posterior to the capitulum) and hypostome (harpoon-like structure forming part of the mouthparts of ticks). Because the posterior end of the hypostome is difficult to locate due to the slope of the tectum, the length measured for the hypostome also includes the base of the capitulum, up to the cornua which are easily identifiable.

Body mass of both unfed and engorged nymphs was measured with an ultra microbalance (Sartorius, Cubis MSA2). Hypostome and scutum lengths were used to divide the nymph population into three initial groups (small, medium and large size; $x_{1,S}$, $x_{1,M}$ and $x_{1,L}$; Fig. 1) with an equal number of individuals. Ticks of each size group were then divided into three treatment lots (Fig. 1; x_2, x_3, x_4), and submitted to the following: (a) DNA extraction and qPCR targeting *Midichloria mitochondrii* on individual unfed nymphs (Fig. 1; $x_{2,S}$, $x_{2,M}$ and $x_{2,L}$),

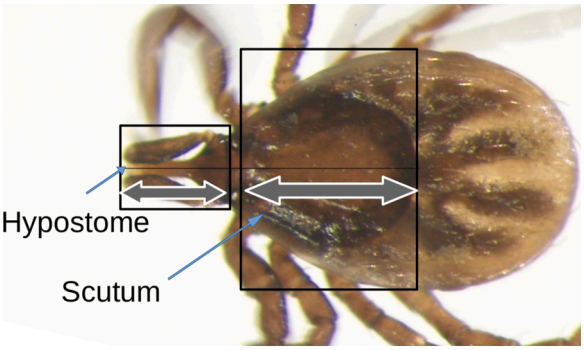

Fig. 1. Experimental design and creation of the size groups ($x_{1,S}$, $x_{1,M}$, $x_{1,L}$). The size groups were each subdivided into two groups, one to measure the *Midichloria* density at the unfed stage (around one third of x_1, $x_{2,S}$, $x_{2,M}$, $x_{2,L}$), the other to perform the blood meal (around two thirds of x_1, $x_{3,S}$, $x_{3,M}$, $x_{3,L}$). The x_3 groups were weighed then divided into two subgroups, one to measure the *Midichloria* density at the fed stage (around half of x_3, $x_{4,S}$, $x_{4,M}$, $x_{4,L}$), the other to determine the sex of the imago (around half of x_3, $x_{5,S}$, $x_{5,M}$, $x_{5,L}$).

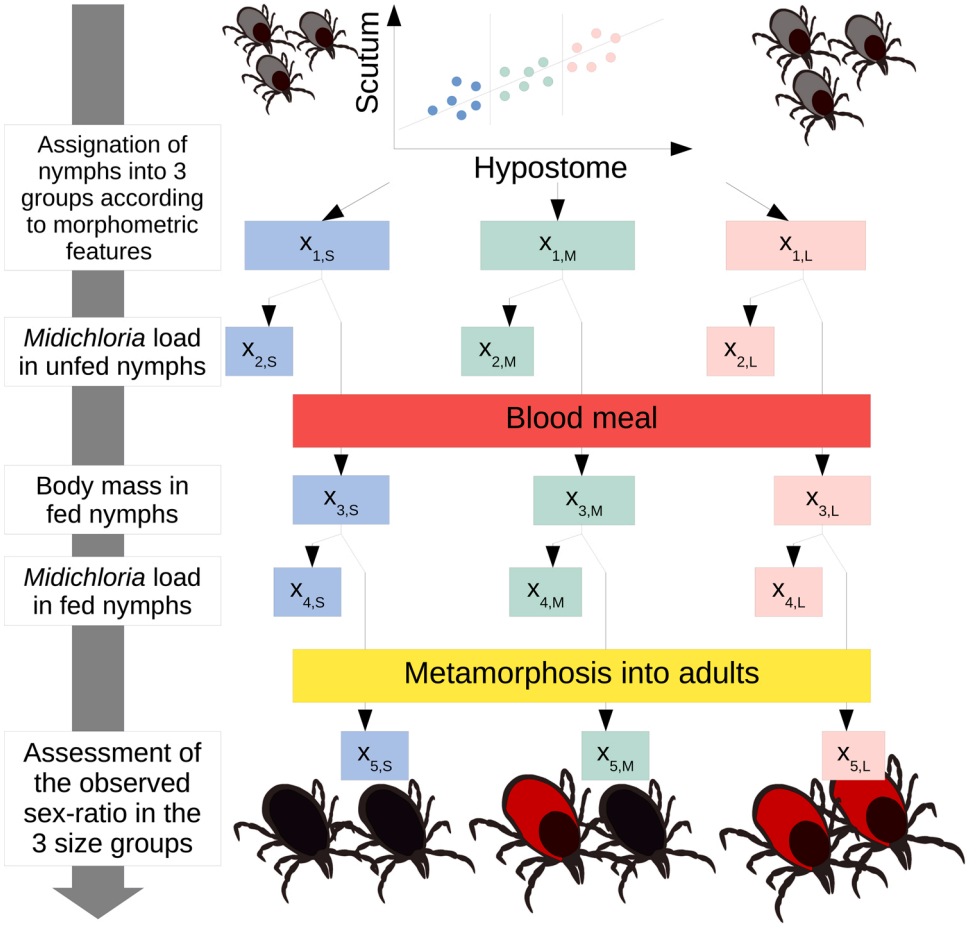

Fig. 2. *Ixodes ricinus* nymph. Hypostome and scutum lengths were measured as indicated by the grey arrows.

engorgement of nymphs on a gerbil (Fig. 1; $x_{3,S}$, $x_{3,M}$, $x_{3,L}$), followed by either (b) DNA extraction and qPCR of individual engorged nymphs (Fig. 1; $x_{4,S}$, $x_{4,M}$, $x_{4,L}$), or (c) moulting to establish sex ratios in each of the 3 size groups (Fig. 1; $x_{5,S}$, $x_{5,M}$, $x_{5,L}$).

2.3. *Ixodes ricinus* ticks and morphological measurements

The protocol was approved by the Ethics Committee for Animal Experiments of the Pays de la Loire region (CEEA PdL 06) (Permit Number: 2015−29). Engorgement on gerbils was conducted as described in Bonnet et al. (2007). In short, for each of the size groups (small, medium and large), 47 or 50 nymphs were put on an individual gerbil. The 3 gerbils were each put on a shelf above a tray of water, in a separate box. Once engorged, ticks fallen in the water were collected twice a day. It should be noted that the information available for each individual nymph (id est body mass, hypostome and scutum lengths) was lost after engorgement because each tick can not be identified and surveyed individually during engorgement (only the group information is retained).

2.4. Quantitative PCR to determine *Midichloria mitochondrii* density

The ratio between the qPCR quantifications of the symbiont gene gyrase B (*gyrB*) and the tick calreticulin gene (*cal*) allows to estimate the *Midichloria mitochondrii* density, *i.e.* the number of bacteria per tick cell. DNA was extracted using the NucleoSpin Tissue DNA kit (Macherey-Nagel) according to manufacturer's instructions after crushing the ticks using a pestle. As previously described (Sassera et al., 2008), *M. mitochondrii* load was quantified with a SYBR Green Kit (Sigma-Aldrich) using the ratio of single-copy genes - DNA *gyrB* for the bacteria (primer forward CTTGAGAGCAGAACCACCTA and reverse CAAGCTCTGCCGAAATATCTT; amplicon 125 bp) by *cal* gene for the host (primers ATCTCCAATTTCGGTCCGGT and TGAAAGTTCCCTGCTCGCTT; amplicon 109 bp). The results were compared for single nymphs with those of serial dilutions of purified cloned pGEM-T easy plasmid vector (Promega corporation) with known copy number to determine the number of bacteria per host cell at each run. The PCR amplification of the *gyrB* and *cal* genes was as follows: 95 °C for 2 min, 40 cycles at 95 °C for 15 s and at 60 °C for 30 s, and melt curve from 55 °C to 95 °C with increasing increments of 0.5 °C per cycle.

2.5. Determination of the sex ratio in each nymphs group

For each size group (Fig. 1; $x_{5,S}$, $x_{5,M}$, $x_{5,L}$), after engorgement, the nymphs were weighed. Once ranked according to their body mass, one out of two engorged nymph was selected (to form groups with the same body mass distribution) and was maintained in a desiccator with a saturated magnesium sulfate solution (90 % relative humidity) and 20 °C, and checked every day until metamorphosis, at which point their sex could be determined.

2.6. Statistical analysis

All analyses were performed with R (v3.6.2) within the RStudio IDE (v1.2), using ggplot2 (Wickham, 2016), dplyr (Wickham et al., 2020), tidyr (Wickham and Henry, 2020), FSA (Ogle et al., 2020), cowplot (Wilke, 2019) and stats (R Core Team, 2020) packages. The density curves were first built with the built-in density function then using the geom_density function of ggplot2 which uses the kernel density estimation method and relies on the built-in density function implemented

in R. Modes were obtained using a homemade function determining the maxima of each curve. The sample size of each nymphs size group (Fig. 1; $x_{1,S}$, $x_{1,M}$, $x_{1,L}$) was restricted by the maximal number of ticks that can be deposited on an individual gerbil (80 nymphs per gerbil). Moreover, each nymph size group was subsequently split into thirds (Fig. 1; for example the smallsized group was split into $x_{2,S}$, $x_{4,S}$, $x_{5,S}$). Finally, the qPCR method is a destructive method requiring the destruction of individual nymphs (these individuals could not be used to later determine their sex). For all these reasons, the sample size of each group considered is below 30 and thus nonparametric statistical tests were performed.The distribution equality or non-equality was determined using a Kruskal-Wallis test followed by a pairwise Dunn test, or a Wilcoxon rank sum test when only two groups were compared.

3. Results

3.1. Unfed nymphs

3.1.1. Use of hypostome and scutum lengths to build nymph groups exhibiting different sex-ratios

Two criteria - hypostome and scutum lengths (Fig. 2) - were chosen to sort unfed nymphs in three groups (small, medium and large), as shown by the different coloured dots in Fig. 3. Among all measurements, the hypostome measured between 0.500 and 0.733 mm with a mean length of 0.636 ± 0.083 mm whereas the scutum gauged between 1.180 and 1.700 mm with a mean length of 1.494 ± 0.043 mm. These morphometric variables exhibited a correlation ($R^2 = 0.2819$, $P < 0.01$). An equal number of individuals were split into three batches according to their scutum and hypostome length (see Fig. 1; $x_{1,S}$, $x_{1,M}$, $x_{1,L}$; Table 1).

3.1.2. Nymph body mass before engorgement

Before engorgement, the tick body mass showed a normal distribution (102.6–320.1 µg, mean = 199.8 ± 35.3 µg, ±SD), (Fig. S1). Tick body mass was lowest in the small-sized group (mean = 174.5 ± 29.1 mg, $x_{1,S} = 65$) average in the medium-sized group (mean = 203.9 ± 26.8 mg, $x_{1,M} = 65$), and highest in the large-sized group (mean = 221.1 ± 32.8 mg, $x_{1,L} = 65$). Although the 3 distributions were significantly different (Kruskal-Wallis test P < 1e-14), an extensive overlap among the 3 distributions is observed.

3.1.3. Symbiont density in unfed nymphs

In unfed nymphs, *M. mitochondrii* density fluctuated from no bacteria detected to 5.63 bacteria per host cell (mean 7.3e-01 ± 1.21) (Fig. 4). The *gyB/cal* ratio of the small-sized nymphs (mean = 0.58 ± 1.39, $x_{2,S} = 18$) presented the highest heterogeneity of symbiont density. Values were more uniform for the large-sized nymphs (mean = 0.85 ± 1.01, $x_{2,L} = 15$), with the *gyrB/cal* ratio clearly close to 1 bacterium per cell (except for one outlier) (Fig. 4). Medium-sized nymphs (mean = 0.77 ± 1.24, $x_{2,M} = 15$) appeared to cluster in two density groups, one around 0.001 and another around 1 bacterium per host cell. At this unfed nymph stage, the symbiont densities of the 3 size groups were not significantly different: none of the size groups displayed a symbiont density higher than the other (Kruskal-Wallis test, P = 0.16).

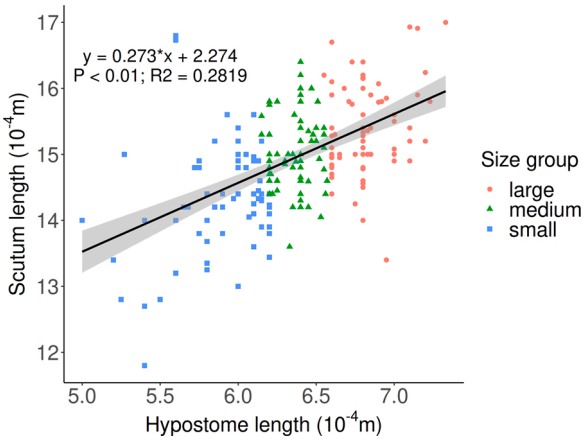

Fig. 3. Composition of the three groups (small, medium, large) based on the hypostome and scutum length, measured at the unfed stage. Blue dots - small ($x_{1,S}$), green dots - medium ($x_{1,M}$), red dots - large ($x_{1,L}$). (R2 = 0,2819, P < 0.01). The unit used for scutum and hypostome lengths is 10^-4 m (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2. Fed nymphs

3.2.1. Determination of sex ratio in each nymph size group

After moulting of individual engorged nymphs of known body mass, we obtained males and females for each size group as described in Table 1. Mortality rate was not found to be significantly different between each size group. The small-sized group exhibited a majority of males (11 males, 2 females) whereas the large-sized group exhibited a majority of females (15 females, 3 males). It should be noted that although the sex-ratios are different among the 3 groups (*i.e.* 85 % *vs* 17 % of males; test of given proportion, p-value < 0.001), both the small-sized and the large-sized groups contained individuals of both sexes. Regarding the body mass of engorged nymphs, nymphs that became male were significantly lighter than nymphs that became female (Fig. 5; Wilcoxon rank sum test P<1e-08).

After having merged all three size groups, the distribution of the body mass density displayed a bimodal pattern, with two clearly distinct modes (mode 1 = 2.79 mg; mode 2 = 4.75 mg) (Fig. 6). These last results allowed us to introduce a new variable, the predicted sex, determined according to the body mass of the engorged nymphs. Therefore, we inferred the predicted sex of each engorged nymph based on its body mass considering a threshold value between the two sexes set to 3.78 mg. This threshold corresponded to the minimal value observed between the two distributions (see the vertical line in Fig. 6).

The observed body mass of engorged nymphs for the small-sized group ranged from 2.16 to 5.36 mg, with a mean of 3.12 ± 0.84 mg (Fig. 7), exhibiting a bimodal distribution (Fig S2a) with the first (major) mode around 2.70 mg (25 individuals; predicted sex male) and the second (minor) mode around 4.60 mg (6 individuals; predicted sex female).

The body mass for the medium-sized group ranged from 2.28 to 6.21 mg (Fig. 7) (mean = 3.81 ± 1.01 mg) with a bimodal distribution (Fig. S2b). The first mode corresponded to 18 individuals (mean body mass: 2.90 mg; predicted sex male) while the second mode corresponded to 18 nymphs (mean body mass: 4.60 mg; predicted sex female), with thus a balanced size of the 2 groups corresponding to the 2 modes.

The body mass for the large-sized group ranged from 2.48 to 6.73 mg (mean = 4.55 ± 1.03 mg) (Fig. 7), with a bimodal distribution (Fig. S2c), the first (minor) mode with a mean around 2.90 mg (8 nymphs; predicted sex male) and the second (major) mode around 4.80 mg (29 nymphs; predicted sex female).

Without considering those bimodal distributions for each size group, the median size was 2.84 mg for the small-sized group, 3.6 mg for the medium-sized group and 4.78 mg for the large-sized group. Those 3 medians of the different size groups were significantly different (Kruskal-Wallis test, P < 1e-06), the comparisons small-medium, medium-large and small-large were also significantly different (post-hoc Dunn's test, P-values respectively below 1e-03, 1e-03 and 1e-07).

The density curves based on the individuals with a predicted sex clearly correspond to two gaussian distributions with different parameters (male mode = 2.74 mg; female mode = 4.79 mg) (Fig. S3).

3.2.2. Symbiont density in fed nymphs

A total of 106 (72.1 %, $n = x_{3,S} + x_{3,M} + x_{3,L} = 147$; Fig. 1) nymphs

Table 1
Number of nymphs in the three different size groups (small, medium, large) and their use in the different investigations (qPCR for part of the unfed and fed nymphs, moulting to the adult stage to determine the sex-ratio in each size group for the remaining part of fed nymphs).

Group	qPCR unfed	Failed to feed	qPCR fed	Moulted to adults = $x_{5,S/M/L}$		Died during moulting	Total
				Female	Male		
Small (S)	$x_{2,S} = 18$	16	$x_{4,S} = 16$	2	11	2	$x_{1,S} = 65$
Medium (M)	$x_{2,M} = 15$	13	$x_{4,M} = 19$	8	8	2	$x_{1,M} = 65$
Large (L)	$x_{2,L} = 15$	12	$x_{4,L} = 19$	15	3	1	$x_{1,L} = 65$
Total	$x_2 = 48$	41	$x_4 = 54$	25	22	5	$x_1 = 195$

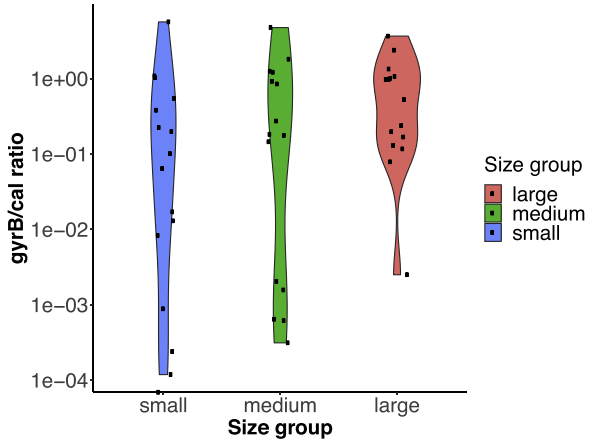

Fig. 4. Ratio gyrB/cal indicating the *Midichloria mitochondrii* density, in the different unfed nymph groups.

Fig. 5. Body mass of engorged nymphs. Sex determination of those nymphs corresponded this time to the observed sex as it was based on the sex of the adults obtained after moulting of engorged nymphs (that were weighed prior to metamorphosis).

engorged successfully. Among those 106 engorged nymphs, a subset of 54 (16, 19 and 19 from the small, medium and large-sized groups respectively; Fig. 1; $x_{4,S}$, $x_{4,M}$ and $x_{4,L}$) were crushed, DNA was extracted and the symbiont density was quantified.

The *gyrB/cal* ratio for the small-sized group ranges from 2.2e-04 to 1.9, with a mean of 0.34 ± 0.59 (Fig. 8, S4a), exhibiting a bimodal distribution with the first mode around 1e-02 (11 individuals, predicted sex male; one *gyrB* value was null and cannot therefore be included in the figure) and the second mode around 1 (5 individuals, 3 predicted sex female and 2 predicted sex male). The ratio for the medium-sized group ranges from 5.6e-03 to 2.0 (Fig. 8, S4b) (mean = 0.69 ± 0.73), with a bimodal distribution, the first being around 1e-02 (8 individuals, predicted sex: male) and the second one being around 1 (8 individuals,

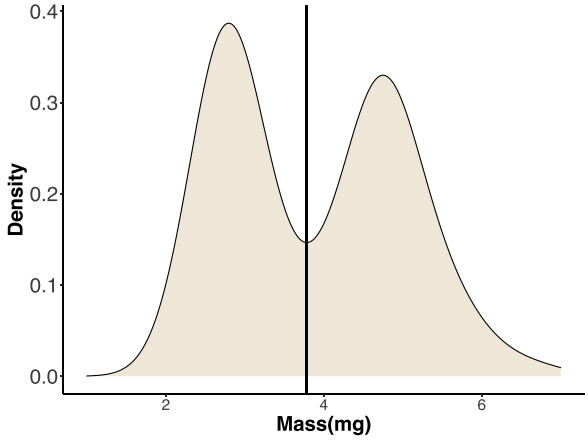

Fig. 6. Density estimator of the body mass (a) with all engorged nymphs from the 3 groups (small, medium, large) merged. The vertical line indicates the minimum between the two modes (body mass = 3.78).

predicted sex: female). The *Midichloria* density for the large-sized group ranges from 1.8e-03 to 7.6 (mean = 2.3 ± 2.6) (Fig. 8, S4c), and exhibits a first mode at 1e-02 (3 individuals, predicted sex: male) and a second mode at 1 (16 individuals, predicted sex: female, except for one individual). The three groups were significantly different (Kruskal-Wallis test, P-value = 0.01), however only the small and large-sized groups were significantly different (post-hoc Dunn's test, P-value = 0.0054).

The body mass displayed a significant correlation with the log10 of *Midichloria* density (Spearman's rank correlation, rho = 0.8249104, P<1e-11) (Fig. 9). Two distinct groups can be recognized, with the first one (blue ellipse in Fig. 9) corresponding to nymphs with the smallest body mass (predicted sex: male) and exhibiting the lowest gyrB/cal ratios, while the other cluster (red ellipse) gathered nymphs with the highest body mass (predicted sex: female) and the highest *gyrB/cal* ratio.

4. Discussion

4.1. Validation of groups of unfed nymphs with different sex-ratios based on morphometric features

To compare the growth of *M. mitochondrii* during engorgement in nymphs that will become either males or females, it is necessary to identify *a priori* (*i.e.* before engorgement) the sex of nymphs, using a non-destructive method (allowing future engorgement). Our strategy to build 3 groups of unfed nymphs based on morphological features (using scutum and hypostome lengths, as suggested by Dusbabek et al., 1996) and exhibiting different sex-ratios was successful. Following engorgement, we validated that the sex-ratios among the 3 groups (initially formed according to those morphological features) were significantly different. The differences in sex-ratio among the 3 groups (of unfed nymphs *i.e.* $x_{1,S}$, $x_{1,M}$ and $x_{1,L}$) were also confirmed by the engorged nymphs body mass ($x_{3,S}$, $x_{3,M}$, $x_{3,L}$) where the sample size of each of the two modes (corresponding to males [the lightest] and females [the heaviest] respectively) are clearly different among the 3 groups (Fig. 7

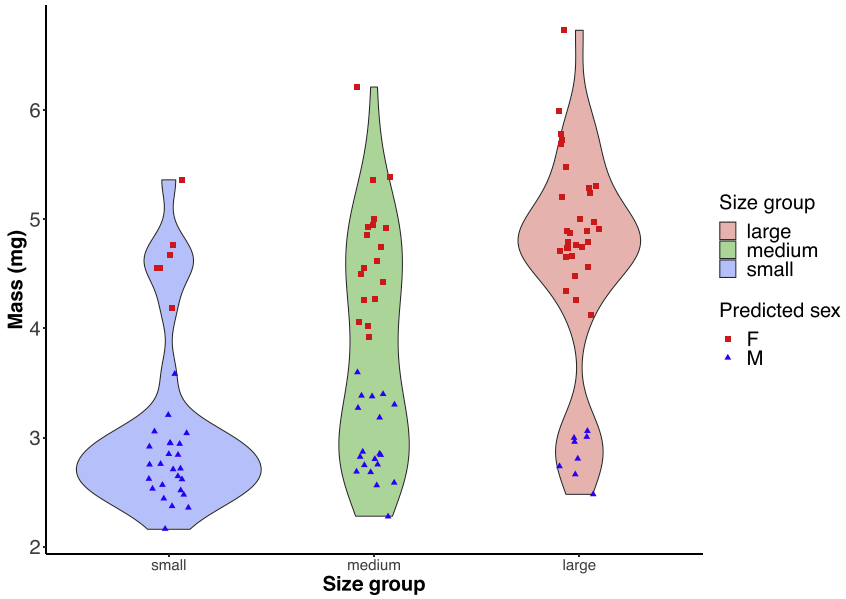

Fig. 7. Violin plot of the body mass according to the fed nymph groups.

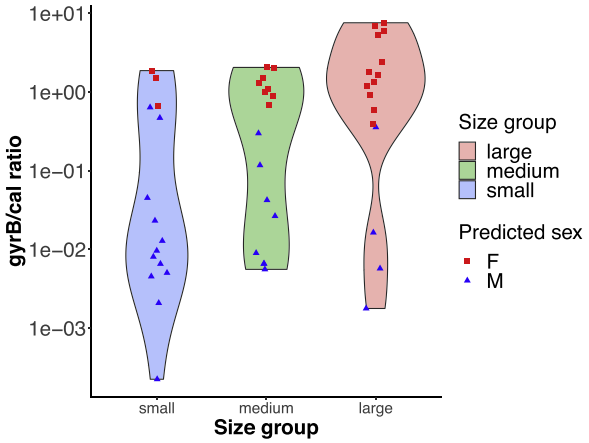

Fig. 8. Individual gyrB/cal ratios of fed nymphs in the three size groups.

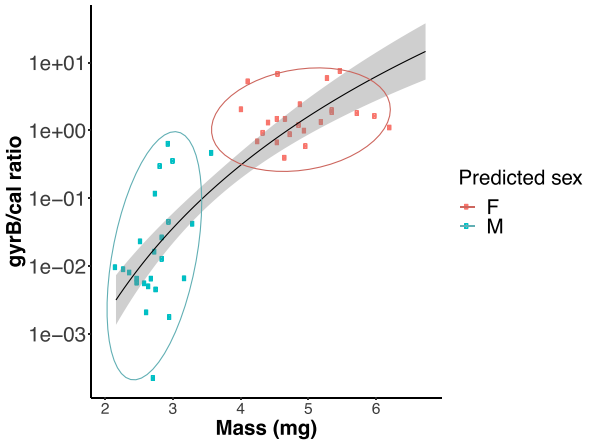

Fig. 9. Relationship between fed body mass and *Midichloria* density (gyrB/cal ratio) for fed nymphs of *I. ricinus*. Ellipses were made using the stat_ellipse() function of the ggplot package.

and S2). Indeed, while the overlap between the body mass distribution of unfed nymphs is too broad to allow sex determination (as it did not exhibit a bimodal pattern; Fig. S1), once engorged, nymphs that will become females are significantly heavier than nymphs that will become males, with no overlap between the two body mass distributions observed where the highest body mass for an engorged male-to-be nymph was 3.60 mg (n = 22) and the smallest body mass for an engorged female-to-be nymph was 3.92 mg (n = 25) (Fig. 5). This last observation confirms previously published results on *I. ricinus* (Dusbábek et al., 1995, 1994; Kahl et al., 1990). Similar observations have been made on other tick species (*I. rubicundus* (Belozerov et al., 1993), *I. scapularis* (Hu and Rowley, 2000), *Amblyomma americanum*, *A. maculatum*, *Dermacentor variabilis*, *Rhipicephalus sanguineus* sensu lato (Nagamori et al., 2019)), however this is not strictly the case for all tick species (see for instance *D. andersoni* (Nagamori et al., 2019). Although the differences based on morphological features and engorged body mass between nymphs that will become either males or females were already known (Dusbabek et al., 1996), we are not aware of any studies on ticks published to date that have been able to build groups exhibiting different sex-ratios before engorgement. This strategy that we have validated through our experimental approach can now be used to investigate not only symbiont load in sexually immature stages, but any other biological traits that would aim to compare nymphs that will become males with those going on to become females.

4.2. Use of Midichloria load in individual nymph to determinate their sex

Regarding unfed nymphs, there are no significant differences in *Midichloria* load between the three groups of nymphs (Fig. S1). Thus, the overlap in *Midichloria* load between nymphs that will become males and those that will become females is too extensive to predict the sex of adult *I. ricinus*. Firstly, the reduced sample size of our data set may partly explain the absence of significant differences observed among the 3 groups. However, even with a larger sample size, it would be difficult to assign a sex to an individual nymph with a high level of confidence, because intermediate values of the *gyrB/cal* ratio (*e.g.* 1e-2) are observed in the small-sized group of nymphs (Fig. 4). Secondly, the lack of significant differences among the 3 groups of unfed nymphs could be due to the heterogeneity in symbiont load among individual nymphs within a given sex, as suggested by the wide range of *gyrB/cal* ratios observed within a given group (Fig. 4). This inter-individual variability may be

explained by differences in primordium development among individuals at such an early stage of the development of the gonad tissue.

Concerning fed nymphs, even if there is a clear difference in *Midichloria* load between the nymphs that will become males and those that will become females (see the ellipses in Fig. 9), there is still an overlap of the 2 distributions of endosymbiont density. The addition of the information corresponding to engorgement body mass is thus needed to predict whether an individual nymph will become a male or a female. This may be due to interindividual variations in *Midichloria* load prior to engorgement (as observed in Duron et al., 2018). Even if the engorgement is clearly responsible for an increasing difference in *Midichloria* load according to sex, those differences are not sufficient to identify the sex of an individual nymph.

4.3. Evolution of Midichloria load in nymphs following engorgement according to their sex

Our study aimed to obtain an accurate comparison of the *M. mitochondrii* density in *I. ricinus* nymphs before and after engorgement and in nymphs that will become either males or females. The rationale was based on the fact that *Midichloria* symbionts are found at a high density in females and are at a very low density - or even completely absent - in males. Until now, only limited data were available to establish the dynamics of this symbiont following the engorgement of its arthropod host. Only the study by Sassera et al. (2008) partially investigated this point and revealed an extensive interindividual heterogeneity of *Midichloria* load, especially in fed nymphs. First, our results clearly demonstrate that *Midichloria* load is significantly different in engorged nymphs that will become males compared to those becoming females, with higher *Midichloria* loads in engorged nymphs that will become females (Fig. 8,9, S4 and S5). The extensive inter-individual heterogeneity of *Midichloria* load observed in fed nymphs investigated by Sassera et al. (2008) could thus be due to the mixture of both males and females, each harbouring a highly different Midichloria load. Those results also suggest that the dynamics of *Midichloria* in its arthropod host are different according to its sex. As with all intracellular vertically transmitted symbionts (Vautrin and Vavre, 2009), males are a dead end for *Midichloria*. Moreover, in the particular case of hematophagous arthropods, as *I. ricinus* males only very rarely bite vertebrates (Balashov, 1972), they probably have limited or null participation in potential horizontal transmission of *Midichloria via* the vertebrate host. Such a horizontal transmission may be possible in the case of females, as suggested by the observation of this symbiont in salivary glands and even in vertebrate blood during an experimental investigation (Cafiso et al., 2018) and also by the incomplete co-cladogenesis between the symbiont and the arthropod phylogenies (Al-Khafaji et al., 2019; Epis et al., 2008; Mariconti et al., 2012). The observed differences in *Midichloria* load between nymphs that will become males *versus* females may thus be due to the different development of gonad primordia (*i.e.* testes in males *versus* ovaries in females). As *Midichloria* is known to be especially abundant in ovarian tissue (Olivieri et al., 2019; Sacchi et al., 2004), the ovarian primordium in fed nymphs that will become females may be more developed than in unfed nymphs and thus may explain the increase in *Midichloria* load between unfed and fed nymphs. Because the difference in Midichloria load between males and females nymphs was observed only in fed nymphs and not in unfed nymphs, we argue that the sole presence of the Y chromosome in males nymphs is not sufficient to explain the observed difference in Midichloria density (otherwise it would also be observed in unfed nymphs). The positive relationship between body mass and *gyrB/cal* ratio observed in Fig. 9 could be interpreted as linear (*id est* "whatever the sex of the nymphs") and resulting from the fact that more metabolite are available in nymphs that will become females due to their larger blood meal. Alternatively, the 2 ellipses in the Fig. 9 (corresponding to the males and females-to-be nymphs respectively) could also be considered as exhibiting each a different slope, hence suggesting a different (non-linear) mechanism involved in *Midichloria* multiplication between the 2 sexes

Like other maternally transmitted symbionts, when located in the somatic line, the bacteria may exhibit a reduced multiplication relative to the one observed in the germinal line. Indeed, only the tissues corresponding to the germinal line contribute to the vertical transmission of the symbiont in its host offspring (Christensen et al., 2019). Nymph dissection to extract ovarian primordia and quantify *Midichloria* in this tissue could be conducted to investigate if there is an increasing density of *Midichloria* in those cells or if the load increases due to the development of these primordia and cell division. Finally, additional investigations, such as the comparison of the dynamics of *Midichloria* in male *versus* female adult tissues (including gonads), before and after engorgement, could also be conducted to provide new information concerning the different fates of the symbiont between the two sexes..

Beside the potential role of endosymbionts as vitamin providers for their arthropod host, it has been recently demonstrated that the endosymbionts *Midichloria* sp., in combination with the *Francisella*-like endosymbionts, are involved in the vectorial competence of *Rickettsia parkeri* by *Amblyomma maculatum* (Budachetri et al., 2018). We thus argue that a better knowledge of the multiplication pattern of endosymbionts in immature stage could also be useful to understand the role of *Midichloria* and its consequences on tick biology as well as the interplay between the symbiont and its arthropod host.

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Author Statement

Romain Daveu: Formal analysis, Writing - Original Draft, Reviewing & Editing, Visualization.

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Declaration of Competing Interest

None

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2021.101707.

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Supplementary material

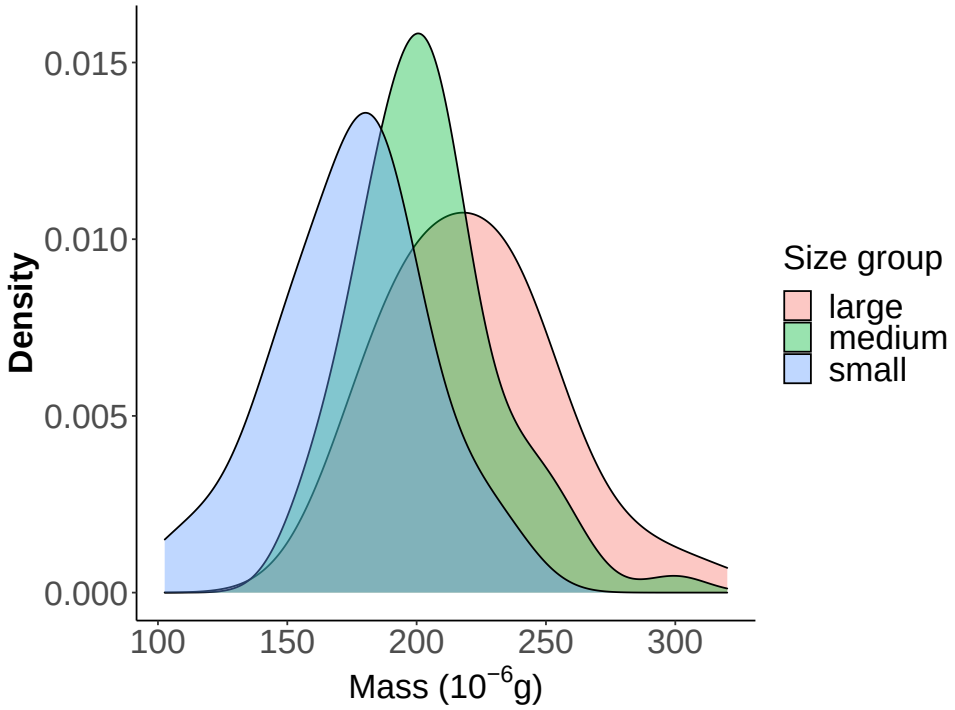

Figure S1 – **Distribution of the body mass for the different size group for unfed nymphs**

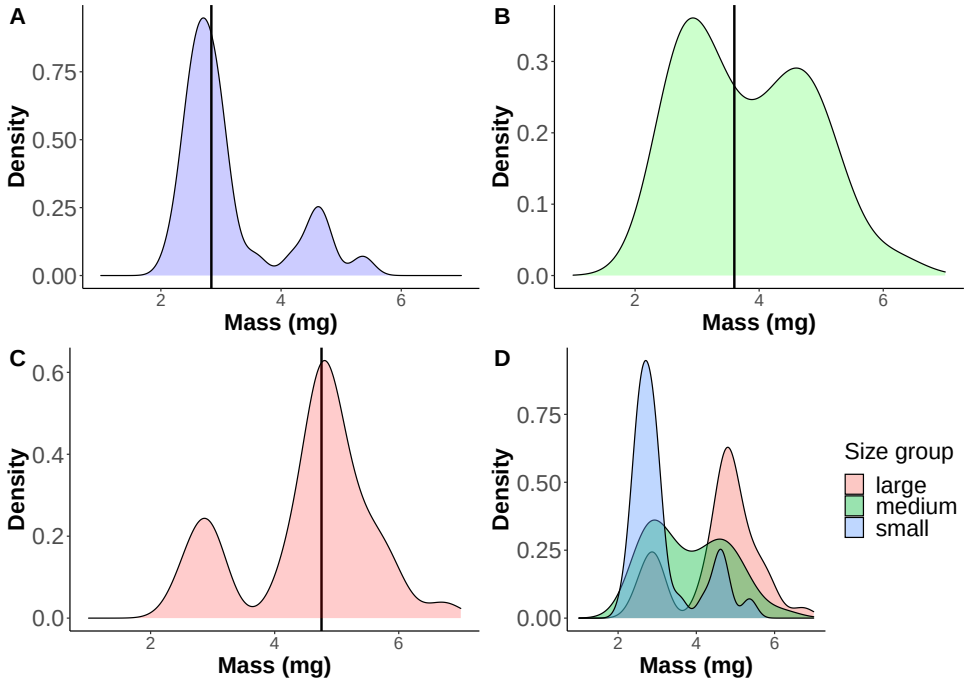

Figure S2 – **Density estimator of the body mass according to the fed nymph groups.** The vertical line indicates the median. (D) corresponds to the superposition of (A), (B) and (C).

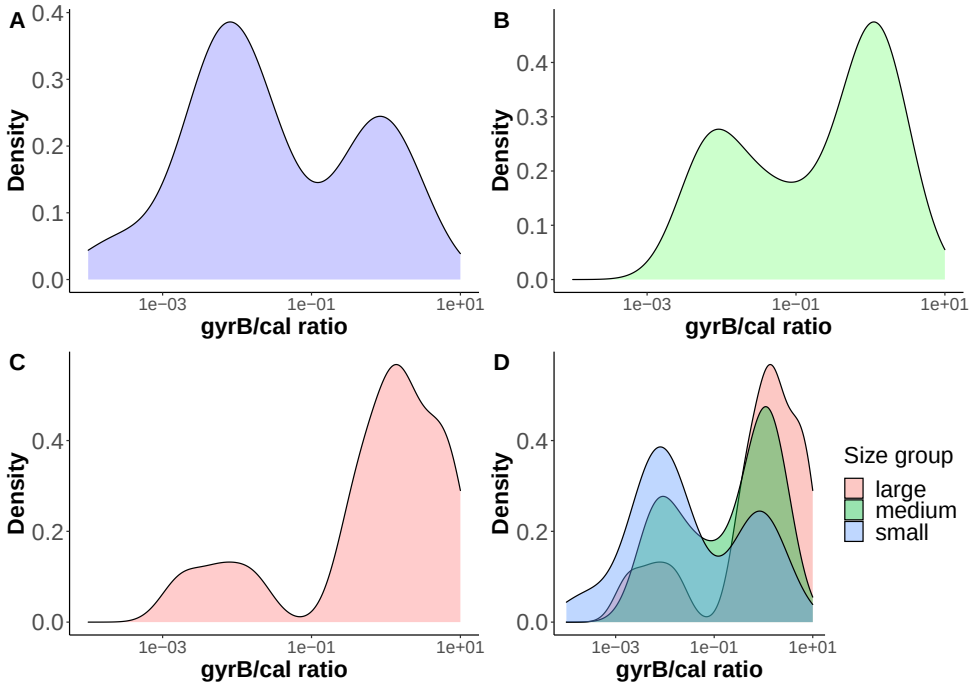

Figure S3 – **Density estimator of the body mass** (a) with all engorged nymphs according to the predicted sex. The vertical line indicates the intersection between the two curves (body mass = 3.78mg).

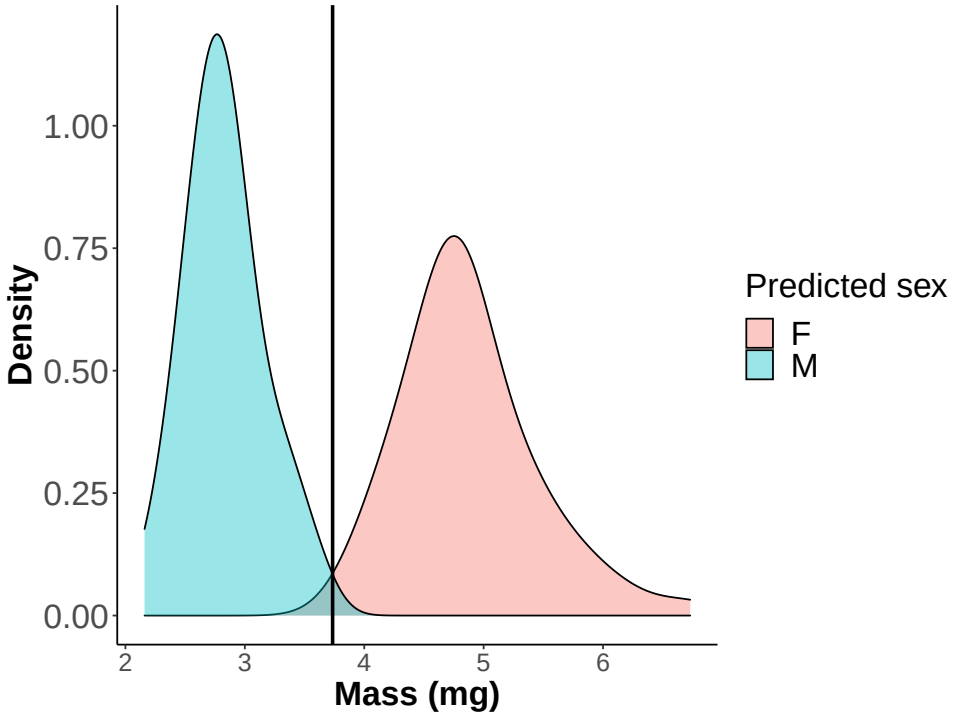

Figure S4 – **Distribution of symbiont density in fed nymphs based on size groups.** A: small, B: medium, C: large, D: all groups together.

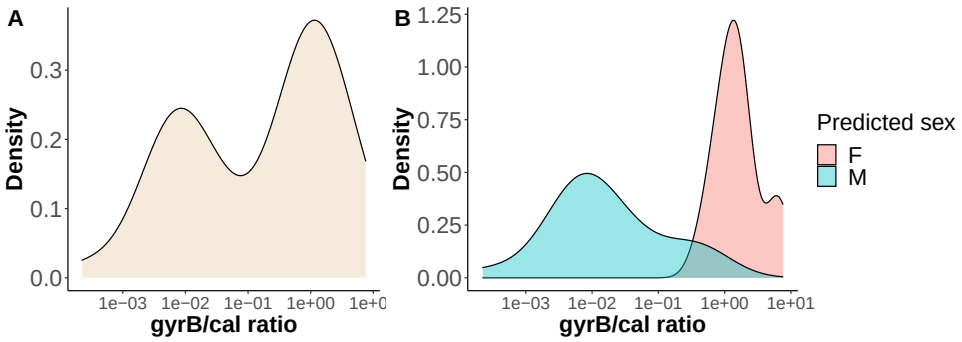

Figure S5 – **Global density estimator of the *gyrB/cal* ratios for the fed nymphs.** A: merged groups. B: according to predicted sex.

Chapter 2

Antibiotics and B Vitamin injections: attempt of *Midichloria mitochondrii* elimination and nutritional rescue

2.1 Foreword

This chapter requires experimental redesign and a larger sample of (surviving) ticks to draw biological conclusions and is not intended for publication in a journal in its present form. However, I have decided to present here in my PhD manuscript the results obtained to discuss the interest of aposymbiotic lines to investigate the interplays between symbiont and host as well as the difficulties to obtain such a biological material.

Context

To investigate the role of a symbiont on the biology of its arthropod host, the use of aposymbiotic (devoid of their symbionts) and wild (without manipulation of their natural microbiome) strains to compare various biological features (life-history traits such as fecundity or life-expectancy, survival after exposure to pathogens/parasites, reproduction, nutrition, emergence rate) are especially useful. The availability of such aposymbiotic strains have had a prominent importance in the understanding of the various and complex interactions between symbionts and their host (Oliver et al., 2009a). However, producing such aposymbiotic strains may not be an easy task. This is notably the case for ticks. Those arthropods are strictly hematophagous, exhibit a long life-cycle lasting two to three years in the wild and request three different blood meals on three different hosts to complete their development and produce a progeny. Although the use of antibiotics is

a frequently used tool to cure arthropods from their symbionts, the case of *Midichloria mitochondrii* is rather delicate. As (i) these bacteria cannot be grown on artificial media (to date, no tick cell line harbouring these bacteria exists; the lines described in Najm et al., 2012 are harbouring bacteria related to *M. mitochondrii* but not this particular species strictly speaking; Sassera, pers. comm.) and (ii) *Ixodes ricinus* cycle, like many ticks, is long, the assays and tests of different antibiotics to select the most effective one is particularly challenging. Moreover, the use of artificial feeding systems to feed ticks turn out to be hardly compatible with the availability of a control tick strain (harbouring its symbionts). Indeed, if antibiotics are not used, contaminations of the blood and the membrane are hardly avoidable within the artificial feeding system. The apparatus also needs to be kept running during one or two weeks to allow tick engorgement, which is significantly longer compared to other blood feeding arthropods (Bonnet and Liu, 2012). Usually, the feeding success rate is lower and the feeding duration is longer compared to *in vivo* feeding (Bonnet and Liu, 2012). Moreover, for obligate symbionts — a characteristic that may be considered compatible with the nearly 100% prevalence of *M. mitochondrii* in wild *I. ricinus* females —, aposymbiotic strains are expected to not survive (or barely), unless a supplementation corresponding to the symbiont metabolic supply is provided (by adding B vitamins in the diet for example; Duron et al., 2018; Hosokawa et al., 2010).

Objectives

The first objective of this work was to create an aposymbiotic line of I. ricinus devoid of *M. mitochondrii* (notably for further use in Chapter III) by nano-injecting the tetracycline antibiotic. In addition, to test if *M. mitochondrii* is indeed a B vitamin provider for its arthropod host, as shown or suggested for other symbionts of other tick species, a part of the ticks was also injected with B vitamins together with the antibiotic for potential nutritional rescue (plus, other ticks were injected with B vitamins only, for control). Two concentrations of antibiotics and B vitamins have been tested, leading to a combination of nine treatments (with the injection water control). Life-history traits of all those treatments were compared to assess the involvement of *M. mitochondrii* on tick biology and its potential nutritional role.

Main results

A few days after the injections of the different treatments within flat *I. ricinus* females, an unexpected high mortality rate has been observed, especially for the antibiotic and B vitamin treatments with the highest concentrations. This mortality rate drastically reduced the sample size preventing any statistical significance for the various life-history traits recorded. Among the measured life-history traits, the mass of engorged females and the egg mass seemed to be lighter compared to the control and the other treatments for the highly dosed antibiotic treatment. However, no apparent loss or density variation of *M. mitochondrii* was observed in the progeny of these injected females. Several methodological issues (which could have been detrimental to tick survival or *M. mitochondrii* elimination from the ticks) prevented us from drawing any conclusion about the phenotypic effect of *M. mitochondrii* presence on *I. ricinus*. To increase the chance of success of such a experiment, it could be useful to test the choice of a different localization for the injection jointly with the usage of a micromanipulator to improve nano-injection accuracy, the use of a different antibiotic such as ciprofloxacin (in comparison with several others), as well as the use of the less concentrated dose for the B vitamin mixture, to successfully remove *M. mitochondrii* and to prevent any excessive premature death of ticks due to the chemical properties (e.g. pH, molarity) of the different treatments to demonstrate the role of *M. mitochondrii* on tick fitness and the possible nutritional rescue by B vitamins.

2.2 Introduction

Following the discovery that arthropod endosymbionts are extremely widespread, almost to ubiquity, and that they are influencing a large number of biological features of their host (including nutrition, survival against natural enemies, vector competence...), there is an increasing interest for tick endosymbionts. In certain cases, maternally inherited symbionts have become obligate mutualists retaining or acquiring the capacity to synthesize important biochemical products in order to be essential to their arthropod hosts (Moran et al., 2008; Wernegreen, 2012).

Ticks endosymbionts belong to at least ten maternally inherited bacterial genera, namely *Arsenophonus*, *Spiroplasma*, *Rickettsia*, *Rickettsiella*, *Wolbachia*, *Cardinium*, *Lariskella*, *Coxiella*-like, *Francisella*-like and *Midichloria* (Bonnet et al., 2017; Bonnet and Pollet, 2020; Duron et al., 2017). To date, the three latter (as endosymbionts) have only been found in ticks and not in any other arthropods (Bonnet et al., 2017). Although the percentages may vary according to the authors, pieces of evidence show that around 50% of ticks are infected by *Coxiella*-like endosymbionts (CLE) and 20% by *Francisella*-like endosymbionts (FLE) (Duron et al., 2017). Therefore, *Midichloria* endosymbiosis with ticks is rarer compared to CLE and FLE, but is still relatively common as the bacterium is found in many tick species (9 out of 17 species investigated in Cafiso et al., 2016; 32 out of 81 species investigated in Duron et al., 2017; 8 out of 21 species in Epis et al., 2008). In addition, *Midichloria* prevalence varies strongly within different species but can be close to 100% (at least in females and progeny) in the European species *Ixodes ricinus* (Guizzo et al., 2020; Lo et al., 2006), the Australian species *I. holocyclus* (Beninati et al., 2009; Chandra and Šlapeta, 2020; Gofton et al., 2015) or the African species *I. aulacodi* (Cafiso et al., 2016) for instance. Moreover, the bacterial diversity found in the ovary of *I. ricinus* was found to be very low, with *M. mitochondrii* being the dominant endosymbiont, and with ovarian microbiome exceeding the midgut 16S rDNA copy numbers by several orders of magnitude (Guizzo et al., 2020).

The study of the endosymbiont effects on obligate blood-feeder biology by the creation of aposymbiotic lines using antibiotics is a method used for several decades (Nogge, 1976). More recently on ticks, Zhong et al., 2007 paved the way to demonstrate the concrete influence of CLE on reproductive fitness of *Amblyomma americanum*, by direct injection of

antibiotics on fully engorged nymphs. Years later, several studies followed, using different antibiotics, different methods (injections at various stages of the tick life cycle or using an artificial feeding system), targeting different symbionts in different tick species (Ben-Yosef et al., 2020; Duron et al., 2018; Guizzo et al., 2017; Li et al., 2018) while measuring different fitness parameters. However, some publications did not necessarily succeed to demonstrate the mutualistic relationship (for instance with a fitness improvement) between a given symbiont and its tick host. For example, antibiotic treatment to delete *Rickettsia* endosymbiont of *I. pacificus* was shown not to influence the incubation period and egg hatching rate (Kurlovs et al., 2014), while an effect could have been observed after blood meals and/or in subsequent stages such as nymphs or females. Similarly, an attempt to remove *M. mitochondrii* has been made on fully engorged females of *I. ricinus* using tetracycline (Ninio et al., 2015). In this case the treatments did not allow to eliminate the symbiont population from the host tick, but only to reduce the multiplication that occurs after the larval blood meal. The presence of *M. mitochondrii* in the offspring of treated females could be explained by the bacteriostatic nature of tetracycline, which probably only prevents *M. mitochondrii* multiplication. Moreover, as *M. mitochondrii* density is known to dramatically increase after repletion (Sassera et al., 2008), conducting antibiotic injections prior to the engorgement, rather than after the engorgement, could be much more efficient to prevent *M. mitochondrii* multiplication in the ovary.

As ticks are obligate blood-feeders that rely on blood as their unique external source of nutrients, this diet is unbalanced, with high proportions of proteins and iron, relatively low lipids and carbohydrates, and not containing enough vitamins and cofactors essential to ticks for basic metabolic reactions necessary to complete their life cycle. Therefore, like many other arthropods feeding on a biased diet, ticks have developed during evolutionary times intimate relationships with different microorganisms (endosymbionts) that supply the missing nutrients (Akman Gündüz and Douglas, 2009; Kirkness et al., 2010; Nikoh et al., 2014; Sassera et al., 2013).

In order to test if *M. mitochondrii* is indeed a supplier of B vitamins to the host as some other endosymbionts have been proven to be, ticks were nano-injected with either (i) antibiotics [for *M. mitochondrii* suppression/growth inhibition], (ii) B vitamins [supplementation control], (iii) antibiotics and B vitamins [nutritional rescue] or (iv) water control. Several objectives were at stake, especially to produce an aposymbiotic *I. ricinus*

strain (notably for further use in Chapter III) and to compare life-history traits to assess the involvement of *M. mitochondrii* in tick biology.

2.3 Material and methods

Ticks

Two hundred forty-four wild *I. ricinus* female ticks were collected on the vegetation in Carquefou, France in April 2018, along with ca. 200 males. Ticks were maintained in desiccators with 90% humidity (using a saturated magnesium sulfate solution), at 4°C in dark. One week before their use, ticks were put at 20°C 12 hrs dark:12 hrs light in order to reactivate basic metabolic functions. Ticks were individually weighed with an ultra microbalance (Sartorius, Cubis MSA2), and equally distributed for each treatment.

Antibiotic treatment

Choice of the antibiotic

Tetracycline was the antibiotic selected because it was reported to be effective against Rickettsiales, notably against the phylogenetically related Alphaproteobacteria (Anaplasmatacea) *Wolbachia* (Bandi et al., 1999; Dedeine et al., 2001). It should be noted that to date, all the five publications reporting success to cure ticks of their endosymbionts were targeting two Gammaproteobacteria, *i.e. Coxiella*-like endosymbionts (gammaproteobacteria) (Ben-Yosef et al., 2020; Guizzo et al., 2017; Zhang et al., 2017; Zhong et al., 2007) or *Francisella*-like endosymbionts (Duron et al., 2018). Those two bacterial genera have closely related bacterial species known to be pathogenic for humans (or animals) (Duron et al., 2015; Gerhart et al., 2016; Gerhart et al., 2018) and thus, previous pharmacological investigations have allowed identifying efficient antibiotics. For endosymbionts that can not be grown outside tick cells such as *Midichloria* no efficient antibiotics have been identified to date. As no tick cell line infected with *Midichloria* exists to date (but see Najm et al., 2012), antibiotic efficiency tests are limited, considering previously published works, as well as its mode of action (bacteriostatic which limits bacterial multiplication in contrast to bactericide which kills bacteria), its dosage and the dilution buffer.

Injection within ticks

In total, 234 flat ticks were injected using a NanoInjector (Drummond) (n = 26 females for each condition). The injected treatments were (i) water, (ii) antibiotic solely (two doses, see below), (iii) B vitamins solely (two doses, see below), or (iv) antibiotic and B vitamins (four treatments by the combination of each of the two doses), resulting in a total of nine treatments to ensure homogeneity among treatment groups. Antibiotic treatment and vitamin supplementation were done using the same methods, and all treatments were prepared under a laminar flow hood to insure sterility. Due to the impossibility for the capillary glass to directly penetrate the cuticle without breaking it, a hole in the cuticle was conducted prior to the injection using an entomology minuten pin (diameter 0.1 mm, stainless steel) mounted on a mandrel. The piercing was conducted ventrally, in the alloscutum, between the coxae of the leg 4 and the anus, targeting the inner localization of the ovary. This localization was also chosen to be conducted as distant as possible from vital internal organs such as the heart or synganglion (that are located more anteriorly). Ticks were then injected with the adequate treatment in the given hole underneath the cuticle using a nano-injector (Nanoject II Auto-Nanoliter Injector, Drummond) on which a stretched capillary glass was fastened. The injected volume was adapted according to the tick mass so every tick received the same amount of desired product according to their mass. Following the injection, ticks from the same condition were gathered into the same flasks.

Administration of the antibiotic to ticks

In the literature, different administration modes of antibiotics to cure endosymbionts in their arthropod host have been described, including feeding on a host containing antibiotics in its blood, artificial feeding through a membrane on blood containing antibiotics, direct injection of antibiotics in the ticks. The artificial feeding is an attractive method and it has been successfully used in Duron et al., 2018 to delete FLE from *Ornithodoros moubata*. However, soft ticks show a different feeding behaviour compared to hard ticks and feed only during a short period of time (usually several minutes), instead of the long blood meal lasting several days in the case of hard ticks. For this reason, the use of artificial feeding to test the impact of antibiotics in comparison to a control treatment that would not use antibiotics is challenging. Indeed, the use of antibiotics and fungicides is required in order to avoid bacterial/fungal contaminants during the blood meal lasting

several days (as illustrated recently in Militzer et al., 2021). Another option could be to inject antibiotics in the host (*e.g.* rabbits) that would be subsequently ingested by ticks. However, several difficulties linked to (i) the antibiotic degradation in the host, (ii) followed by the variation of antibiotic uptake by the ticks (ticks release most of the serum ingested in the bloodstream, leading to the blood being highly concentrated in the ticks), (iii) additional barriers linked to the digestive tract and the peritrophic membranes around the tick midgut making the antibiotic uptake and the reaching up to the ovary are not in favour of this method. Moreover, ticks being obligate hematophagous acarines, the administration method has to respect the rules of animal experimentation. Some antibiotics, especially when highly dosed, can provoke acute and unnecessary pain and increase uneasiness in the vertebrates used as feeding hosts for ticks. Another possible administration method could have been the use of a capillary (Soares et al., 2005) to allow ticks to ingest the antibiotic solution, followed by the engorgement on the host. However this method seemed more delicate and also raised the issue of accuracy uptake, as mentioned above. The chosen method, antibiotic injections in ticks using a nanoinjector and capillary apparatus requires accuracy and highly concentrated treatment solutions (developed after). Most publications to date have injected antibiotics in fully fed ticks. However, a previous trial conducted with engorged ticks has been unsuccessful in the case of *I. ricinus* (Ninio et al., 2015). Therefore, given that tetracycline is bacteriostatic and *M. mitochondrii* exhibits a multiplication of several orders of magnitude following the tick blood meal, the antibiotic was injected prior to engorgement.

Dose of antibiotics injected

Zhong et al., 2007 and Li et al., 2018 used tetracycline at a concentration of 1.7µg/100mg and 10µg/100mg of engorged tick mass respectively, to treat *Coxiella*-LE or *Rickettsia*-LE from their host. Tetracycline from the cycline family is a bacteriostatic antibiotic that prevents bacteria from reproducing while not necessarily killing them. This antibiotic inhibits protein translation by preventing the binding to the 16rRNA and the binding of the aminoacyl-tRNA to the mRNA-ribosome complex (Chopra and Roberts, 2001; Schnappinger and Hillen, 1996). It has a wide spectrum and is effective against gram-positive and gram-negative bacteria (including *Midichloria*, but also *Borrelia*, *Rickettsia* and *Coxiella*. A flat *I. ricinus* weighs around 1–2mg whereas an engorged female is 100–400mg. Adult female ticks multiply their mass roughly by 100 after engorgement, and most of this mass is made up of concentrated blood. Hence we speculate that after engorgement,

endogenous tick tissues concern solely few percentages of the total mass. The chosen doses were 1500ng/mg (named AB 1X) and 150ng/mg of tick mass (AB 0.1X) (which are around 100-fold less than the doses used for the engorged ticks). Two doses were assayed to maximize the probabilities of success, as one concentration could not have the expected effect or on the contrary could be toxic for ticks. The initial AB 1X solution was at the saturated concentration of 50mg/mL. Water was chosen as the diluent, an attempt to make a 50mg/mL saturated concentration using 1X PBS buffer was unsuccessful.

B vitamin supplementation

B vitamin mixture compositions were prepared according to the ratio of already published studies (Duron et al., 2018; Hosokawa et al., 2010; Lake and Friend, 1968; Nikoh et al., 2014), apart from biotin which concentration was ten-fold multiplied (see Table 2.1 for the concentrations used in the solutions). Indeed, given the possible importance of the biotin supplied by *M. mitochondrii* (Duron and Gottlieb, 2020; Sassera et al., 2011), the low concentration used in the previously cited papers and the will to emphasize the effect of this vitamin, we strongly believed that this increase was necessary.

Choline (B8) and myo-inositol (B4) were historically classified as vitamins but are now considered substrates for enzymatic reactions and can be synthesized by most animals, including insects (Douglas, 2017) and by extension ticks, from common dietary constituents.
Following the same rationale as for the antibiotic solution, two doses (VitB 1X and VitB 0.1X) of B vitamins mixture were prepared with water. Solution solubility was possible only under alkaline conditions. For this reason, solid NaOH had to be added until complete dissolution, (resulting in a high pH of 12 for the VitB 1X solution). The rescue treatments with both antibiotic and vitamins were stirred together notably due to the saturation concentration of the antibiotic solution, resulting in a two-fold dilution of the compounds. Therefore, the injected volume was multiplied by two (increasing injection difficulty with some hemolymph possibly leaving from the tick body after capillary withdrawal).

Infestation and measurement of tick life-history traits

After the injection of the different treatments, several life-history traits were measured as a proxy of tick fitness (Figure 2.1). Five days after injection, the survival rate of the

Compounds	Concentrations (µg/mL) used in Hosokawa et al., 2010	Concentrations of the B vitamins injected in the 1X mixture (ng/mg of unfed tick mass) (VitB 1X)	Concentrations of the B vitamins injected in the 0.1X mixture (ng/mg of unfed tick mass) (VitB 0.1X)
Thiamine (B1)	100	174	17.4
Riboflavin (B2)	20	53	5.3
Nicotinic acid (B3)	100	174	17.4
Pantothenic acid (B5)	100	174	17.4
Pyridoxine (B6)	100	174	17.4
D-biotin (B7)	1	17.4	1.74
Folic acid (B9)	30	52.2	5.22
Cobalamins (B12)	1	1.8	0.18
Choline chloride (former B8)	185	321.6	31.26
Myo-inositol (former B4)	118	205.2	20.64

Table 2.1 – **Composition of B vitamin mixtures used in the literature and for injections**

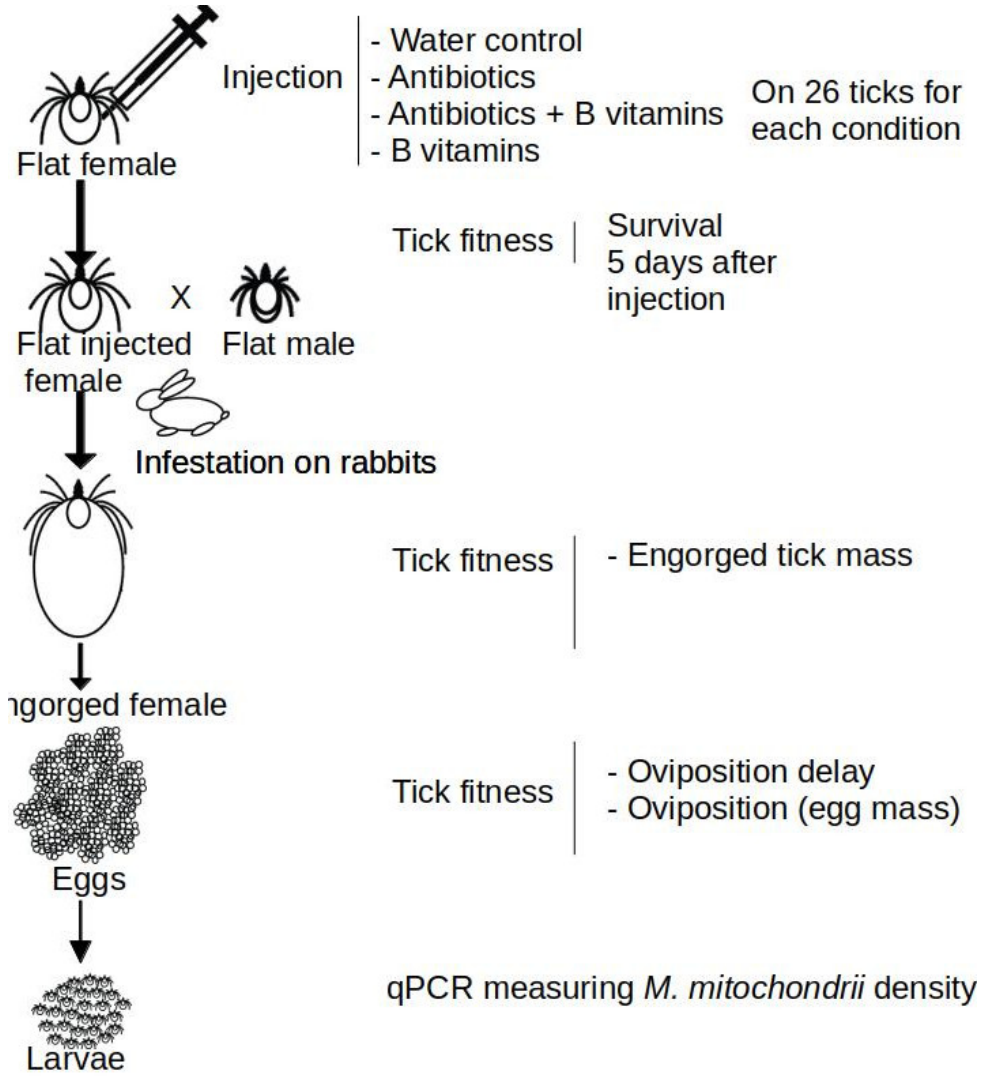

Figure 2.1 – **Experimental design**
Ticks have been submitted to different treatments and tick life-history traits (female engorged mass, egg mass, oviposition delay) are recorded along with *M. mitochondrii* density in progeny

injected females was recorded by testing the tick reaction to CO2 (Table 2.2). Syringes for the deposit on rabbits of the remaining females were prepared at the time as the mating with adult males collected from the same wild population. Meanwhile, a syringe with the ten remaining collected females ticks mated with males acted as a control with no injection. All the ticks were deposited on five New Zealand rabbits (*Oryctolagus cuniculus*) on Tue 5th April 2018 following the protocol described in Almazán et al., 2018 (ethic protocol number: APAFiS #19700), each treatment on a dedicated flank. The fully engorged ticks were recovered between Sun 10th and Fri 15th June 2018, by checking the detachment twice a day. The ticks were immediately measured (height, width, length), weighed and placed into individual sterile 2mL Eppendorf tubes. All deposited ticks succeeded to completely feed and detached naturally. The beginning of oviposition was monitored each day for each female individual. Three weeks after the host detachment when the vast majority of eggs were expelled from the female body, egg clutches were separated from the female body and weighed. These clutches were then placed inside individual flasks until hatching into desiccators at 20°C 12 hrs dark:12 hrs light. The ratio of egg mass by the engorged tick mass was calculated to determine if the related treatment had an influence on the tick capacity to metabolize nutrients obtained from the blood meal for the progeny.

Midichloria mitochondrii titer in progeny

After hatching, ten larvae of each clutch were isolated and subjected to *M. mitochondrii* quantification to evaluate the efficacy of the different injection treatments on the subsequent progeny. As described elsewhere (Daveu et al., 2021b; Sassera et al., 2008, the ratio between the qPCR quantifications of the symbiont gene gyrase B (*gyrB*) and the tick calreticulin gene (*cal*) allows estimating the *M. mitochondrii* density, i.e. the number of bacteria per tick cell. Larvae were rinsed in a bath of bleach followed by two baths of sterile water, to reduce external bacterial contamination (Binetruy et al., 2019b). Then, the pools of larvae were cut in half using a sterile scalpel blade. DNA was extracted using the NucleoSpin Tissue DNA kit (Macherey-Nagel) according to the manufacturer's instructions after crushing the ticks using a pestle. *Midichloria mitochondrii* load was quantified with a SYBR Green Kit (Sigma-Aldrich) using the ratio of single-copy genes — DNA for the bacteria (primer forward CTTGAGAGCAGAACCACCTA and reverse CAAGCTCTGCCGAAATATCTT; amplicon 125 bp) by *cal* gene for the host (primers ATCTCCAATTTCGGTCCGGT and TGAAAGTTCCCTGCTCGCTT; ampli-

con 109 bp). The results were compared for single nymphs with those of serial dilutions of purified cloned pGEM-T easy plasmid vector (Promega corporation) with a known copy number to determine the number of bacteria per host cell at each run. The PCR amplification of the and *cal* genes was as follows: 95 °C for 2 min, 40 cycles at 95 °C for 15 s and at 60 °C for 30 s, and melt curve from 55 °C to 95 °C with increasing increments of 0.5 °C per cycle.

Statistical analyses

All analyses were performed with R (v3.6.2) within the RStudio IDE (v1.2), using ggplot2 (Wickham, 2016), dplyr (Wickham et al., 2020) and tidyr (Wickham and Henry, 2020). Nonparametric tests (Kruskall-Wallis test followed by post-hoc Dunn's tests for paired comparisons) were performed due to the small sample size of each treatment.

2.4 Results

2.4.1 Tick survival following the injection

Twenty-six ticks of each condition were injected with the different treatments (Table 2.2). For each treatment, the survival rate following injection did not exceed two thirds at maximum and was as low as 15.4% or 7.7% for the two most concentrated injections of antibiotic or B vitamins. The control (injection of water in the unfed females) exhibited mortality of 56% illustrating that, independently of the potential toxic effect of antibiotic or B vitamins, the injection was highly deleterious to the survival of the ticks Interestingly, the water treatment induced as much mortality as the [VitB 0.1X] treatment and even more than the [AB 0.1X * VitB 0.1X] treatment. In general, the higher the dose was, the fewer ticks were able to survive, as shown for the sole antibiotic and the sole B vitamin treatments (AB 1X and VitB 1X, respectively). For the rescue treatments (combination of tetracycline and B vitamins), the dose with fewer B vitamins resulted in a higher tick survival rate. Those results suggest that the high concentration of the B vitamins and antibiotics had a negative effect on the survival of injected females.

Injection treatment	Dose	Survival before engorgement (%)	Feeding rate success (%)
H2O (water)		12/26 (46.2%)	12/12 (100%)
Tetracycline (AB)	1X	4/26 (15.4%)	4/4 (100%)
	0.1X	8/26 (30.8%)	8/8 (100%)
B vitamins (VitB)	1X	2/26 (7.7%)	2/2 (100%)
	0.1X	12/26 (46.2%)	12/12 (100%)
Tetracycline (AB) * B vitamins (VitB)	1X * 1X	7/26 (26.9%)	7/7 (100%)
	1X * 0.1X	13/26 (50.0%)	13/13 (100%)
	0.1X * 1X	8/26 (30.8%)	8/8 (100%)
	0.1X * 0.1X	17/26 (65.4%)	17/17 (100%)

Table 2.2 – **Survival rate five days following the injection**

2.4.2 Tick engorgement rate and engorgement duration

All females that survived after the injection succeed to attach to the host following the deposit. Moreover, the engorgement completion was 100% (Table 2.2), for all treatments and the control without injection (10/10). The feeding duration of all females varied between five and 10 days but did not show any significant difference between each treatment, even compared to non-injected ticks (data not shown).

2.4.3 Tick life-history traits

Due to the small sample size number (especially in the treatment of most interest such as antibiotics 1X, 0.1X and B-vitamins 1X, 0.1X), none of the measured tick life-history traits exhibited statistically significant differences, however, trends can be depicted.
The tick mass after engorgement did not differ between any of the treatments, except for the AB 1X treated ticks (but note the reduced sample size, with four individuals surviving in this treatment). Three out of the 4 AB 1X females weighed around 2-fold less compared to the water control and the other treated ticks (Fig. 2.2A). The same pattern appears for the egg mass of those AB 1X treated ticks. In addition, while the engorged mass of the two Vitamin B 1X ticks seems similar compared to the other treatments (except AB 1X) (Fig. 2.2A), their egg masses are three times inferior in comparison to the H2O control

(Fig. 2.2B). This tendency is shown while examining the ratio of egg mass by engorged tick mass (Fig. 2.2C). The rest of the ticks, regardless of treatments, seems to produce a total egg mass weighing around half of their total engorged mass (with few exceptions to this pattern).

All fully engorged ticks were able to lay eggs. Some extensive variation in the time from detachment to oviposition was found for the AB 1X ticks, some starting to lay relatively late compared to the control (Fig. 2.2D). Moreover, the two VitB 1X ticks laid later compared to the other treatments.

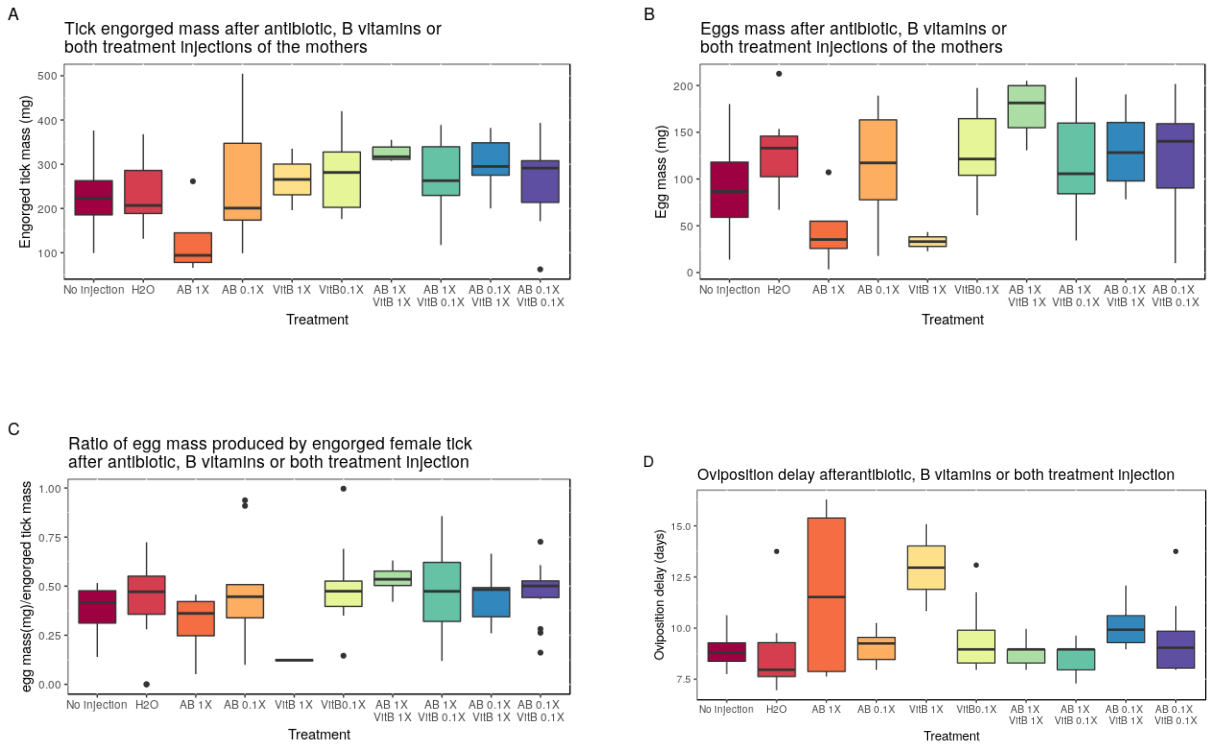

Figure 2.2 – **Tick life-history traits following the injection (i) water, (ii) tetracycline, (iii) B vitamins or (iv) tetracycline and B vitamins.**
A: Engorged tick mass. B: Egg mass obtained from laying females. C: Ratio of egg mass by engorged tick mass. D: Delay (in days) before the beginning of oviposition of each female.
No injection: ticks from the same wild population that were not submitted to injection; H2O: water injection. AB 1X: tetracycline 1X; AB 0.1X: tetracycline 0.1X; VitB 1X: vitamin B 1X, vitB0.1X: vitamin B 0.1X; AB 1X VitB 1X: tetracycline 1X * vitamin B 1X; AB 1X VitB 0.1X: tetracycline 1X * vitamin B 0.1X; AB 0.1X VitB 1X: tetracycline 0.1X * vitamin B 1X

2.4.4 Effect of antibiotic and B vitamin treatments on *M. mitochondrii* density in progeny larvae

Using qPCR, the *M. mitochondrii* titer in progeny larvae has been investigated on four selected treatments (including the control — injection of water —, the treatment with the highest antibiotic dose — AB 1X —, the treatment with the lightest B vitamin dose — VitB 0.1— and the corresponding complementation treatment — AB 1X * VitB 0.1X —; Fig. 2.3). *M. mitochondrii* density was not significantly reduced in AB 1X (n=4) and AB 1X * VitB 0.1X (n=13) or VitB 0.1X (n=12) treatments compared to the water control (n=12) and none of the batches was completely negative for *M. mitochondrii* (except one sample among the 12 of the VitB 0.1X treatment; Fig. 2.3).

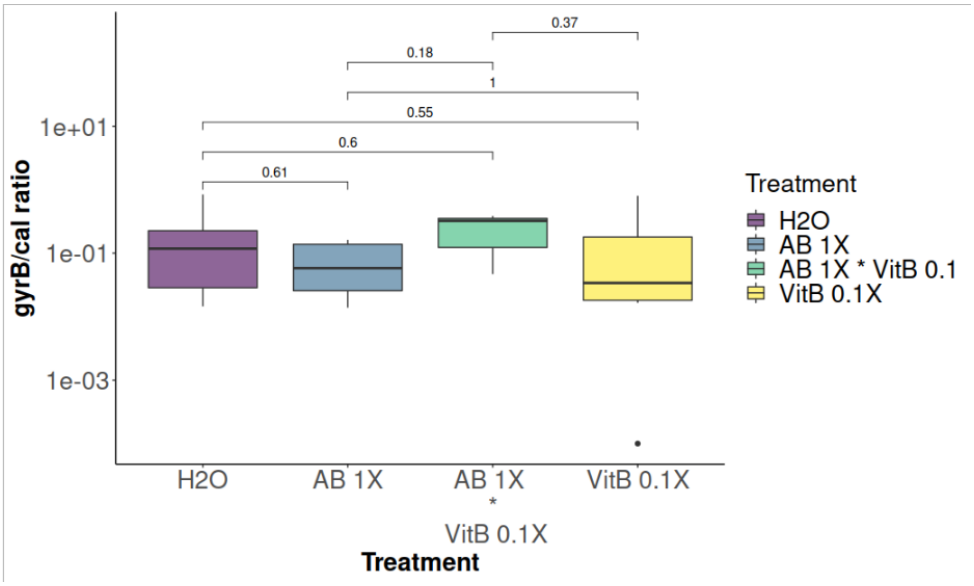

Figure 2.3 – ***Midichloria mitochondrii* density of the progeny of the females injected with the different treatments.**
H2O: water injection. AB 1X: tetracycline 1X; AB 1X * VitB 0.1X: tetracycline 1X * vitamin B 0.1X; vitB0.1X: vitamin B 0.1X. Values above the boxplot indicate p-values of pairwise comparisons obtained after a Dunn's test.

2.5 Discussion

2.5.1 Mortality rate observed after injection

A large mortality rate was observed following injection (56% in the case of the control treatment corresponding to water injection; 93% for the treatment exhibiting the highest mortality). Besides natural mortality (ticks were collected in the field, without any knowledge of their age or potential factors affecting their survival), the observed mortality may be due to (i) the destruction of internal organs during injection, (ii) loss of hemolymph following piercing, or (iii) bacterial/fungal infection following the introduction of foreign bodies within the tick internal cavity. The use of a micromanipulator could potentially reduce such a phenomenon by improving handling accuracy, instead of handling the nanoinjector apparatus with one hand. Indeed, the handling time of the nanoinjector with the capillary within the tick body being increased with higher volume injected as it requires pressing several times on the injection button of the nanoinjector, in addition to the resting time inside the tick body to ensure the good delivery of the different solutions. Even though the minuten used for the piercing was quickly sterilized by flame sterilization between each injected tick to sterilize its surface, it was not possible to do the same with the capillary glass between each injection, due the glass melting that shuts the capillary hole at the tip. Thus, bleach sterilization (followed by rinsing twice with sterile water) was performed for the glass capillaries. Moreover, the hole produced in the tick tegument may not have been properly filled (following the withdrawal of the capillary glass, due to cicatrisation of the tegument and cristallisation of hemolymph), even if we expected that it should be less problematic in the case of injection in flat females than in fully engorged females (as usually conducted for injection in ticks in most studies). The hole sealing would occur several days after, while it should be hampered by the extensive enlargement of the tegument following the rapid feeding phase when injection is performed in engorged females. If part of the injected liquid (either water, antibiotic or vitamins) have flowed out of the tick body due to a lack of filling of the hole following piercing, it may also have impacted the volume of injected products (antibiotic and/or vitamins) and thus their effect.

It should be noted that the only treatments for which a negative impact was observed regarding the life-history traits (AB 1X and VitB 1X; Fig. 2.2A, B and C), corresponding to the injections with the highest concentration, were also the treatments with the highest mortality.These suggest a certain toxicity at those concentrations. However, according to

this hypothesis, the treatment combining AB 1X and VitB 1X should also exhibit a high mortality rate and negative impact on life history traits, but this was not observed. It should be noted also that the AB 1X solution had a very low pH (close to 1, due to tetracycline-HCl form) while the VitB 1X solution was highly alkaline (following sodium hydroxide addition). Those extreme pHs are potentially a cause for the high mortality rate for the highly concentrated treatments. Therefore, the mix of both AB 1X and VitB 1X solutions produces a more neutral pH, likely explaining a mortality rate that was not as dramatic as expected.

As for the water treatment, the non-negligible mortality rate could be due to the osmotic deregulation disturbing tissues and cells. Compared to other works such as Ben-Yosef et al., 2020, no mortality was observed following injection, perhaps because they use PBS buffer instead of simply water. Otherwise, the other papers do not necessarily mention the injection success rate.

2.5.2 Antibiotic efficacy

As mentioned earlier, besides the dose, the nature of the antibiotic and its potential efficacy is crucial to eliminate *M. mitochondrii* from its host. Despite tetracycline seemed a legitimate choice, being in continuity with the work of Ninio et al., 2015, we did not observe any negative effect on *M. mitochondrii* density. Its efficacy against the phylogenetically related bacteria *Wolbachia* (*e.g.* Bandi et al., 1999) or, in another context of another tick symbiont, against *Coxiella*-like endosymbiont (Li et al., 2018; Zhong et al., 2007) made it an ideal candidate. The density of *Rickettsia* endosymbiont of *I. pacificus* was slightly affected by tetracycline in eggs laid by the treated mother, but ciprofloxacin was shown to be more effective (Kurlovs et al., 2014). The suppression of *R. buchneri*, the most frequent *I. scapularis*, was also a success when the ticks were injected with ciprofloxacin (Oliver et al., 2020). Another potential source of information is that we could take advantage of antibiotic sensibility of culturable *Rickettsia* (which are also phylogenetically close to the *Midichloria* genus). For instance, *R. felis* was found to be susceptible to doxycycline, rifampin, thiamphenicol, and fluoroquinolones (Rolain et al., 2002). It is worth noting that we succeeded in removing *M. mitochondrii* using marbofloxacin (in order to create an aposymbiotic line), although very few ticks survived (see Foreword of Chapter III). The measure of life-history traits on this particular line was delicate due to the low sample size at our disposal. In addition, the B vitamin provider hypothesis required to perform the same experimental design as the ticks submitted to the marbofloxacin treatment, which

was not feasible.

2.6 Conclusion

Several methodological issues (notably leading to mortality or preventing the elimination of *M. mitochondrii* from the ticks) prevented us from drawing any conclusion about the phenotypic effect of *M. mitochondrii* presence on *I. ricinus*. We advise for further research the choice of a different localization for the injection jointly with the usage of a micromanipulator to improve accuracy, the use of a different antibiotic such as ciprofloxacin (or the comparison with several others), as well as the use of the 0.1X dose for the B vitamin mixture, to successfully remove *M. mitochondrii* and to prevent any excessive premature death of ticks due to the chemical properties of the different treatments to demonstrate the role of *M. mitochondrii* on tick fitness and the possible nutritional rescue by B vitamins.

Chapter 3

Multilayered transcriptomics in the hard tick *Ixodes ricinus* comparing feeding stages, multiple organs, and symbiotic status

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Manuscript in preparation (to be submitted in a modified and more concise form — notably the result section which have been too expanded).

3.1 Foreword

Endosymbionts of arthropods are known to impact numerous biological functions of their host, including notably nutrition (and hence development), reproduction or immune response (Haine, 2008; Moran et al., 2008; Welchman et al., 2009; Wernegreen, 2012). To identify the biological functions influenced by the presence or absence of a symbiont, a transcriptomic approach is especially relevant as it allows an integrative investigation taking into account simultaneously all the expressed genes (or transcripts) by this organism. Regarding ticks, even if there are on-going or released whole-genome sequencing projects (Gulia-Nuss et al., 2016; Jia et al., 2020; Murgia et al., 2019), the functional annotation of tick genes is still limited and no annotated genome of *Ixodes ricinus* is currently available. The main question behind this work was to characterize the transcriptomic response of *I. ricinus* in the presence or absence of *Midichloria mitochondrii*, to better understand the interplay between the host and the symbiont. In order to reduce the complexity of the system and help the interpretation of the data on differential gene expression, we have decided to consider separately three different organs:

- the ovary which is the organ where *M. mitochondrii* exhibits the highest density

and thus a key organ to understand the interplay between this bacterium and its arthropod host (Beninati et al., 2004; Olivieri et al., 2019; Sacchi et al., 2004).

- the Malpighian tubules which are known to harbour some endosymbionts that have been revealed to be obligate symbionts in other tick species (Buysse et al., 2019; Duron et al., 2018; Klyachko et al., 2007 and where *M. mitochondrii* has been detected but in a significantly fewer quantity (Olivieri et al., 2019)

- the salivary glands which are the best-known organ for tick transcriptomic (e.g. Garcia et al., 2014; Martins et al., 2019; Perner et al., 2018; Ribeiro and Mans, 2020; Schwarz et al., 2013), allowing comparison with previous studies, including change in gene expression due to the presence of particular pathogenic bacteria.

Moreover, as the blood meal is known to have a tremendous effect on the expression of numerous tick genes — in connection with the extensive development of those three organs after feeding — (Karim and Ribeiro, 2015; Rudenko et al., 2005; Sonenshine and Roe, 2013) and on *M. mitochondrii* explosion (Sassera et al., 2008), this transcriptomic investigation has been conducted both on unfed and partially-fed *I. ricinus* females.

Context

Given the intimate relationship in which *I. ricinus* and *M. mitochondrii* are associated, the first difficulty we had to face was to obtain an aposymbiotic strain of *I. ricinus*, free of *M. mitochondrii*, or at least with a significantly reduced titer of the bacterium. A secondary constraint was to minimize the differences between the symbiotic and the aposymbiotic strains to assess with maximal confidence that the observed differences between the two transcriptomes were due to the symbiont presence or absence, e.g. the genetic variability and the environmental conditions had to be as low as possible. The most straightforward path would be to collect a wild *I. ricinus* strain harbouring *M. mitochondrii* and to remove the symbiont for half of those ticks. In that aim, we chose to perform antibiotic injections. As discussed in Chapter II, little is known regarding the antibiotic efficacy against the *Midichloria* genus. Tetracycline has been tested in engorged *I. ricinus* females but did not lead to complete elimination of the endosymbiont (Ninio et al., 2015; Chapter II). Some other attempts have been tried in the lab before the beginning of my thesis, notably with marbofloxacin. Flat F0 females collected from the wild have been injected either with marbofloxacin (MARB) or either with 1X phosphate-buffered saline solution (PBS, as

a control) as soon as June 2015. Unfortunately, a high mortality rate led to only one F1 MARB female remaining in February 2018. To obtain a sufficient number of females, another F2 generation was started up. Although the tick life cycle duration can be shortened in some way under laboratory conditions compared to the life cycle in the wild, we encountered enormous delays regarding the moult between each stage compared to what we were used to (four to six months instead of around one month usually). Meanwhile, *M. mitochondrii* density has been quantified at each developmental stage (according to the method described in Sassera et al., 2008). The MARB lineage was confirmed to have an undetectable amount of symbiont and the PBS lineage maintained a comparable titer of *M. mitochondrii* compared to what has been described in the literature (Sassera et al., 2008).

In the frame of this thesis, we were lacking time regarding the emergence of F2 adults. Nonetheless, the creation of *I. ricinus* line devoid of *M. mitochondrii* appears feasible, indicating that the presumed obligate relationship between *I. ricinus* and this symbiont should be treated with caution, despite apparent high costs on the fitness of this aposymbiotic lineage as evidenced by the developmental delays and high mortality rate observed.

As we were unable to produce a new tick line following antibiotic injection (Chapter II) and as the aposymbiotic line (MARB) and its control line (PBS) produced in the lab were not available in sufficient number in time to perform the experiment, we used an ancient lab strain (more than 30 generations in the lab) from the University of Neuchâtel, Switzerland, kindly provided by Dr Maarten Voordouw. This strain has lost *M. mitochondrii* as the generations went by. The phenomenon of progressive *M. mitochondrii* lost in lab strains over generations has already been mentioned in the literature (Lo et al., 2006). For the purpose of this work, this lab strain has been compared to a wild strain harbouring *M. mitochondrii*, collected in Chizé, France.

Methods

Organs (namely ovary, Malpighian tubules and salivary glands) were cautiously dissected either after mating without any engorgement (D0 time point) either four days after mating and deposit on the vertebrate host (D4 time point) for both strains (namely Wild — for wild ticks from Chizé, France — or Lab — for the laboratory strain from Neuchâtel, Switzerland —). Total RNA from each sample was extracted and sent to the company

Novogene, Hong-Kong, for RNA-sequencing by poly(A) selection. Obtained raw reads were trimmed and controlled for quality. A meta-transcriptome assembly was built based on six independent assemblies (by organ and time point) and annotated against different databases. Gene expression was assessed following alignment and read counting against the different transcripts. Exploratory analysis of library patterns was carried out. Differential expression analysis was performed for each tissue independently, by comparison of the feeding stages and symbiotic status. This step was followed by Gene Ontology (GO) enrichment and gene set testing analyses to assess functional patterns. Significantly differentially expressed genes with extreme log-fold changes were occasionally considered individually, notably when no GO term association was found (which count for ca. 50% of the assembly).

Results and discussion

The measured metrics (notably the number of BUSCO genes) indicate that the transcriptome assembly quality is excellent. The PCA plots show that the clusterization of the libraries appears evident for the organs followed by the feeding stage. When each organ is individually considered, the libraries cluster by feeding stage and symbiotic status. Globally, the differential expression analyses revealed much more differentially expressed genes for the feeding stage comparisons compared to the symbiotic status comparisons (around 10-fold more). Regarding the feeding stage comparisons, the Biological Process (BP) GO term "protein phosphorylation" was systematically enriched for the mid-engorged ticks regardless of the organ. For the first time, the main enriched biological functions are characterized for the Malpighian tubules at two time points of the tick life cycle, in particular genes encoding several sulfotransferases at the unfed stage with the highest log fold-changes and the Molecular Function (MF) GO term associated (sulfotransferase activity) being particularly significantly enriched. The ovary describes an enrichment for biological roles notably related to signal transduction and responses that could be interpreted as the preparation of dramatic morphological changes. Regarding the symbiotic status comparisons, around twice as many genes are differentially expressed at D0 compared to D4. The presence of *M. mitochondrii* in the ovary at mid-engorgement provokes an enrichment of the BP GO categories referring to 'activation of NF-kappaB-inducing kinase activity' and 'positive regulation of JNK cascade', which could be potentially interpreted as a trigger of the tick innate immune system, notably through the activation of 6 undetermined "RING-type domain-containing protein" or "Putative TNF receptor-

associated factor" (TRAF) genes. TRAFs are notably intermediate components of the Toll pathway, (Fogaça et al., 2021). This pathway could cause the synthesis of antimicrobial peptides (AMP). A gene encoding an AMP annotated as "ricinusin" was the most or one of the most differentially expressed genes in terms of log fold-change in the presence of *M. mitochondrii* whichever comparison considered. Still in the ovary but in the non-gorged stage, the enrichment of the GO MF term "heme binding" in the wild-type strain (thus carrying *M. mitochondrii*), could be related to heme biosynthesis by *M. mitochondrii* (Sassera et al., 2011). Heme, which is toxic at high doses, is predominantly derived from the blood meal and ticks lack the ability to synthesize it (Perner et al., 2016b). This cofactor is stored in specialized organelles (hemosomes, within intestinal cells; Lara et al., 2003) and distilled sparingly for cellular needs, it is not excluded that *M. mitochondrii* may provide a non-negligible amount of this cofactor in the ovary during the unfed stages of *I. ricinus*. Additionally, the Cellular Component (CC) GO term "TIM22 mitochondrial import inner membrane insertion complex" was enriched for both unfed and partially-fed time points for the ovary of the symbiotic lines. The two genes related with this term are annotated as "Mitochondrial import inner membrane translocase subunit Tim29" and are potentially targets to understand the subtleties related to the peculiar mitochondrial tropism of *M. mitochondrii*. Although these hypotheses need to be explored experimentally, these new transcriptomic data provide new insights into the involvement of these different genes/biosynthetic pathways regarding *M. mitochondrii-I. ricinus* interactions.

3.2 Introduction

Ticks are hematophagous ectoparasites responsible for the transmission of important diseases both in humans and animals (Jongejan and Uilenberg, 2004), and are classified in two main families displaying two different feeding behaviours. In particular, the ca. 700 hard ticks species feed on vertebrate hosts thrice, once for each immature stage, larva and nymph, to reach adulthood (Balashov, 1972; Guglielmone et al., 2014); the last blood meal of adult females lasts approximately 6-12 days according to the different species, and is needed to provide nutrients for egg production (Balashov, 1972). *Ixodes ricinus* is the most widespread and important tick in Europe as its distribution area goes from Scandinavia to North Africa, from Portugal to Russia (Medlock et al., 2013) This species is widely studied due to its medical importance, as the pathogens it carries and is able to transmit as a obligate blood feeder, such as *Borrelia burgdorferi s.l.* responsible for Lyme disease and the TBE virus causing tick encephalitis, are widespread and harmful (Parola and Raoult, 2001a). Beside pathogens, ticks are also hosts of maternally transmitted symbiotic microbes which have been shown to have significant effects on the arthropod phenotype (Ahantarig et al., 2013; Bonnet et al., 2017; Narasimhan and Fikrig, 2015; Narasimhan et al., 2021). *Candidatus* Midichloria mitochondrii (hereafter *Midichloria mitochondrii*) is believed to be the most frequent vertically transmitted symbiont of *I. ricinus* (Aivelo et al., 2019; Guizzo et al., 2020; Sassera et al., 2006), with a fixation rate close to 100% for females collected in the field, eggs and larvae (Beninati et al., 2004; Lo et al., 2006), but being progressively lost possibly at the nymphal stage (Daveu et al., 2021b). This intracellular bacterium presents two tropisms in the oocytes of its host: part of the population is cytoplasmic, while a significant portion of the symbionts displays the peculiar feature of being able to reside in great numbers between the inner and the outer membrane of mitochondria (Lewis, 1979; Sacchi et al., 2004; Zhu et al., 1992). *Midichloria mitochondrii* density inside the tick body explodes upon engorgement, especially in the ovary (Olivieri et al., 2019; Sassera et al., 2008). In addition to the ovary, *M. mitochondrii* populations appear to be non-negligible in Malpighian tubules and salivary glands, but less abundant than in the ovary by several orders of magnitudes (Olivieri et al., 2019). Salivary glands are the home of horizontally transmitted pathogens within ticks, and key organs for Ixodida due to their role during engorgement, where a cocktail of molecules is released inside the host to bypass the immune system of the vertebrate host (Sauer et al., 2000). Given their crucial importance, salivary glands are the most studied organs in transcriptomic

investigations in ticks (Ribeiro and Mans, 2020). Malpighian tubules are considered to be important organs for osmotic regulation, detoxification and excretory functions (production of nitrogenous waste) but their fine role in tick biology remains poorly characterized (Sonenshine and Roe, 2013). In the case of some tick-symbiont associations (mainly with *Coxiella*-like endosymbionts and *Francisella*-like endosymbionts) they have been reported as a key organ to host the symbiont, sometimes with symbiont aggregates as what could be compared to insect bacteriocytes (Buysse et al., 2019; Duron et al., 2018; Guizzo et al., 2017; Klyachko et al., 2007).

Following studies performed in other symbiont-host association (Burke and Moran, 2011; Emery et al., 2017; Mann et al., 2017; Smith and Moran, 2020), we used RNA-sequencing to investigate the host transcriptome and study, for the first time in ticks, the impact of a symbiont on tick gene expression with the goal to better understand the effect of *M. mitochondrii* on its host. We address this unprecedented question by comparing gene expression in two tick lines, a wild one and an aposymbiotic one, within three different organs (mainly the ovary, but also salivary glands and Malpighian tubules) at two time points of the tick life cycle (before and during feeding).

3.3 Material and Methods

3.3.1 Tick samples

Wild-type (hereafter WT) repleted nymph ticks were collected from roe deer in Chizé Forest, Chizé, France (46.14677, -0.42493) in February 2018. Those nymphs were left to moult and maintained in a desiccator with a magnesium sulfate solution (90% humidity, 20°C) until further use. A laboratory strain (from now on, Lab) has been reared for ca. 30 generations in the University of Neuchâtel, Switzerland. Prior analyses (unpublished) indicated that this specific *I. ricinus* strain had a highly reduced titer of *M. mitochondrii* (confirmation described below). Regarding the maintenance of the Lab line in the laboratory, in short, individuals of each stage (larva, nymph, adult) were allowed to feed on mice, and the mating step was done only with males from the same colony. For our generation of interest, nymphs were fed on naive rabbits using a previously described protocol (Almazán et al., 2018) (ethics protocol number: APAFiS #19700).

For both lines, engorged nymphs were recovered and placed into a specific desiccator (90% humidity, 20°C) until the moulting step was achieved. Fifteen unfed mated adult females were then collected from both lines and named D0 (day 0; before the deposit on the host), while 15 mated females from both lines were allowed to engorge. Female engorgement of the two lines took place in Oniris Vet School, Nantes, France, in March 2019 using a single rabbit for each. The 15 semi-engorged mated females were manually removed from the host four days after the deposit on rabbits and named D4 (day 4) . Removal was performed using a tick twister, and ticks were individually put in Eppendorf tubes and left chilled on ice to freeze development and gene expression until dissection, which occurred within minutes from the removal.

3.3.2 Dissection, RNA and DNA extraction and RNA-sequencing

Ticks were dissected on ice to prevent RNA degradation by removing the upper cuticle. For each replicate, five organs (ovary, Malpighian tubules and salivary) were carefully separated, rinsed in chill PBS, pooled before being flash-frozen by liquid nitrogen in tubes containing RNA extraction buffer (LB1 buffer, Macherey Nagel RNA Plus XS kit) and put in a –80°C freezer until further use. Tubes were gently thawed on ice, tissues were mechanically disrupted using decontaminated pestles (3% H2O2) and total RNA was then extracted using the aforementioned kit according to manufacturer instructions. Genomic DNA was trapped inside a specific column, where a small portion of DNA could be retrieved by elution of the Elution Buffer (heated at 70°C) furnished in the Macherey Nagel Nucleospin tissue kit. RNA was sent to the company Novogene (HK) Co., Ltd that processed the quality controls and the RNA sequencing. RNA quantification was performed using a Qubit Fluorometer (Life Technologies) and a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA integrity was measured by a 2100 Bioanalyzer instrument (Agilent). Due to the low RNA quantity in the D0 samples, low-input library preparations were conducted. One of the libraries (D0_N_MT_3, see Table S1 for naming conventions) failed the quality test and was not sequenced. Total RNA was then poly(A) selected and reverse transcribed using either the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (for the D4 samples) or the Clontech Smart-seq v4 ultra low kit (for the D0 samples, which required low-input preparation method). The libraries were sequenced on a Novaseq S4 PE150. with the NovaSeq 6000 S4 Reagent Kit chemistry. Thirty-five samples (three replicates for each tissue, each time point, each strain, except for one

sample corresponding to the D0 Malpighian tubules Lab condition) were sequenced.

3.3.3 Quantitative PCR for *M. mitochondrii*

A previously described method (Sassera et al., 2008) allowed to quantify the abundance of *M. mitochondrii*. In short, quantifications of the symbiont gene gyrase B (*gyrB*; primer forward CTTGAGAGCAGAACCACCTA and reverse CAAGCTCTGCCGAAATATCTT; amplicon 125 bp) and the tick calreticulin gene (*cal*; primers ATCTCCAATTTCGGTCCGGT and TGAAAGTTCCCTGCTCGCTT; amplicon 109 bp) were performed. *Midichloria mitochondrii* density was then expressed by the ratio of *gyrB*/*cal* copy numbers. The results were compared for a single sample with those of serial dilutions of purified cloned pGEM-T easy plasmid vector (Promega Corporation) with a known copy number, to determine the number of bacteria per host cell. The PCR amplifications of the *gyrB*and *cal* genes were as follows: 95°C for 2 min, 40 cycles at 95°C for 15 s and at 60°C for 30 s, and melt curve from 55°C to 95°C with increasing increments of 0.5°C per cycle.

3.3.4 Quality control

Preprocessing of the sequencing reads was done using fastp (Chen et al., 2018). Samples were quality-checked with FastQC (v0.11.9) (Andrews et al., 2010) before and after using Trimmomatic (v0.39) (Bolger et al., 2014) with the following parameters: ILLUMINACLIP:Adapters_Novogene.fa:2:30:10:2:keepBothReads LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15.

3.3.5 *De novo* assembly and annotation

Six independent assemblies by organ (n = 3) and time point (n = 2) were performed using the Trinity software (v2.9.1) (Grabherr et al., 2011) with default parameters. Combining these assemblies, we produced a single reference transcriptome by using the runMeta script from the DRAP software (v1.92) (Cabau et al., 2017), which allows to reduce redundancy and to obtain representative contig sets from multiple assemblies. Assembly completeness was assessed using BUSCO (v4.0.5) (Seppey et al., 2019) using Arthropoda and Arachnida odb10 datasets. Annotation was processed with the Trinotate pipeline (`https://github.com/Trinotate/Trinotate.github.io`; Bryant et al., 2017) using the SwissProt (downloaded on June 22, 2020), UniRef90 (downloaded on Feb 1st, 2020) and *I.*

scapularis genome (Wikel strain, downloaded from VectorBase, v49) databases for blastp and blastx searches. Peptide sequences (and thus CDS) were predicted using Transdecoder (`https://github.com/TransDecoder/TransDecoder`). To retrieve the Gene Ontology (GO) terms, the Interproscan software (v5.44-79.0), based on protein domains and functional site predictions, was used. This strategy is highly relevant to retrieve the most accurate information from the sequence given SwissProt annotation gave on average more generic results compared to the UniRef90 or *I. scapularis* proteins databases.

3.3.6 Mapping and no occurrence of a batch effect

Alignment and read counting were performed with the Bowtie2 (v2.4.1) and RSEM (v1.3.1) softwares (Langmead and Salzberg, 2012; Li and Dewey, 2011) using default parameters. As the libraries were generated in two different runs (one for the D0 and another for the D4) due to the different availability across time of the biological material, the presence of potential batch effects was investigated using the method described in Muller et al., 2019. In brief, raw reads from Charrier et al., 2018 data set, which were obtained from a sequencing on one single lane, were subjected to alignment and read counting against the reference transcriptome as described above. Median-absolute deviation (MAD) values of Counts Per Million (CPM) were calculated for each gene. For this sole analysis, low-expression genes with a chosen threshold of CPM value below 1 were discarded to avoid genes displaying a 0 MAD value (genes being only expressed in one given condition for instance). Genes were sorted in ascending order according to their MAD value. The genes with the 100, 300, 500 and 1,000 lowest MAD values ("low-MAD genes") were checked for potential biases towards highly or lowly transcribed genes and towards short or long genes. Those genes reasonably reflected the distribution of gene transcription and gene length across the entire data set. The same procedure was followed for our data set, and the rank of the 300 low-MAD genes from Charrier et al., 2018 data set was compared to the rank of our total sorted genes. A good proportion of genes was found to be (i) ranked 300 or below, (ii) ranked 1000 or below and (iii) in the top 50% of our data set. Hence, a solid set of genes of low variability regarding expression was shared between the two data sets. We conclude that we did not find any obvious batch effect that clearly differentiates D0 from D4 samples that would impede further analyses.

3.3.7 Differential expression and post-hoc analyses

Downstream differential expression analysis was conducted with edgeR and limma, using the limma-trend statistics (Ritchie et al., 2015; Robinson et al., 2010) for each tissue considered (i.e. ovary, Malpighian tubules and salivary glands). To assess an effect due to the presence of the symbiont *M. mitochondrii*, for the comparisons of WT vs Lab, Lab was used as a reference, as the objective was to establish what are the genes that are differentially expressed (DE) in the WT strain. In parallel, to assess changes due to partial engorgement, for the comparisons of D4 vs D0, D0 was used as a reference. This time, the objective was to establish what are the DE genes upon engorgement, in the temporal order. A gene was considered differentially expressed for an adjusted p-value (FDR) 0.05 and a log2 fold-change (LFC) 2 (meaning a gene is four times more expressed in a condition compared to the other). An exploratory analysis was performed using the Glimma package (Law et al., 2016). DE gene heatmaps were built using the heatmap.2 function from the gplots package (Warnes et al., 2020). Clusters from DE genes were identified using the clust software v1.12.0 (Abu-Jamous and Kelly, 2018). Several approaches were carried out for post-hoc analyses, with two Gene Ontology (GO) enrichment tools (topGO and goseq) and two gene set tests (romer and camera). Of note, using multiple different methods may seem redundant and lead to confusion but for the purpose of this thesis we report them, while only one of each will be retained for the final submitted paper. The topGO package was used for GO enrichment on lists of differentially expressed genes (Alexa and Rahnenfuhrer, 2020), using the weight01 algorithm. As an alternative, goseq package was also used (Young et al., 2010), this software takes advantage of gene length data to correct length biases. Gene set testing statistical hypotheses differ and do not require a significance threshold to be applied to identify differentially expressed genes. Thus, gene set testing was performed based on sets arranged by the previously constituted GO classification to assess if a function was globally differentially expressed or not and in which direction, using the romer and camera functions implemented in the limma package (Ritchie et al., 2015). The romer (ROtation testing using MEan Ranks) function tests a hypothesis similar to that of Gene Set Enrichment Analysis (GSEA) (Ritchie et al., 2015; Subramanian et al., 2005), but uses rotation instead of permutation. The camera (Correlation Adjusted MEan RAnk gene-set test) function tests whether a set of genes is highly ranked relative to other genes in terms of differential expression, accounting for inter-gene correlation (Wu and Smyth, 2012). It is suitable when searching for gene sets of interest and gives more weight to genes that are very strongly differentially expressed.

As a matter of data exploration with another approach using KEGG pathways, the kegga function from the limma package was used for over-representation analysis, with species.KEGG="isc" (for *I. scapularis*) option selected and the *I. scapularis* Wikel strain genome identifiers as input. An other exploratory approach for KEGG pathways was to take differentially expressed gene sequences, extracted at the amino acid level, and then parsed into the BlastKoala server (Kanehisa et al., 2016) to assign KEGG orthologies against *I. scapularis* (taxonomy ID: 6945). The genome of this tick species, from the same genus, is completely sequenced (Gulia-Nuss et al., 2016), its annotation is included in the KEGG database. The exploration was carried out after using KEGG Mapper SearchColor Pathway (`https://www.genome.jp/kegg/tool/map_pathway2.html`). Although this last approach has been partially explored and investigated, the corresponding results are not shown in this manuscript and may not be kept in the final paper.

3.4 Results

3.4.1 qPCR quantifying *M. mitochondrii* in the different organs

To assess the density of the symbiont in the Lab and the WT strains, a qPCR targeting specifically a *M. mitochondrii* gene was conducted. Since the ovary is the organ exhibiting the highest *M. mitochondrii* density relative to the other organs (Olivieri et al., 2019), we only illustrate here the results for the ovary (Fig. 3.1; but see Fig. S1 for the other two organs). *Midichloria mitochondrii* density in the ovary of WT ticks is coherent with the literature (Sassera et al., 2008), confirming the abundant presence of this symbiont in this tissue in our WT strain. However, compared to what observed previously (Olivieri et al., 2019), the blood meal only induced a multiplication by one order of magnitude of the *M. mitochondrii* density in the ovary of WT ticks. This difference with the previously mentioned paper might be partially explained by the fact that the blood meal duration of the semi-engorged ticks was unknown, and possibly longer than 4 days. In addition, we acknowledge that DNA extraction in our work was perhaps suboptimal. Concerning the Lab strain, *M. mitochondrii* could not be detected in D0 libraries. This absence of *M. mitochondrii* in this strain was expected as it has been reared in the Lab for 30 generations and previous studies reported *M. mitochondrii* density to lower from generation to generation once reared under laboratory conditions (Lo et al., 2006). This absence of *M.*

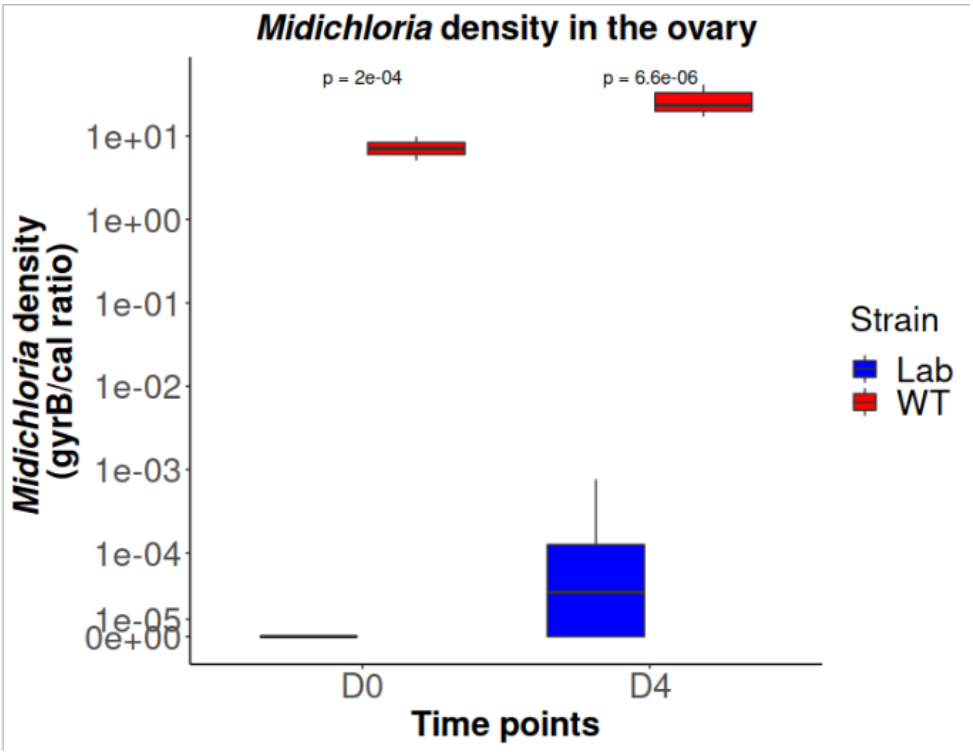

Figure 3.1 – ***Midichloria mitochondrii* titer in the ovary at D0 and D4 time points, in the WT and Lab strains.** The scale is a pseudo-log transformation with sigma = 1e-05, in order to avoid infinite values.

mitochondrii in the Lab strain is also in agreement with our investigations on the deep sequencing of this strain genome (based on DNA extracted from unfed females), where only an extremely reduced number of reads assigned to *M. mitochondrii* were anecdotally retrieved (Rispe, unpublished data). However, at D4, two Lab samples exhibited a non-null *M. mitochondrii* titer (although with an abundance 6 orders of magnitude lower compared to the WT strain). In both the Malpighian tubules and the salivary glands of WT ticks, unexpectedly, the *M. mitochondrii* density was lower after blood meal. Both organs exhibit a conspicuous size growth during the blood meal, tick cell multiplication in those organs could be more important than *M. mitochondrii* multiplication (or *M. mitochondrii* might simply not multiply in those organs), unlike what happens in the ovary. These results reinforce the interpretation that Malpighian tubules and salivary glands are not key organs for *M. mitochondrii* localization and multiplication, as already suggested by their low density in those organs (*gyrB*/*cal* ratio with a maximum at 0.07 compared to 580 in ovary of partially fed females as described in Olivieri et al., 2019). *Midichloria mitochondrii* could not be quantified in the Lab strain at D0. However, unexpectedly, some samples from both Malpighian tubules and salivary glands tested positive at D4, indicating that some *M. mitochondrii* DNA was present. Taken together, these results suggest that *M. mitochondrii* is not totally absent from our Lab strain. However, its density is clearly strongly reduced, even after blood meal (where very high densities are typically reached in WT, especially in the ovary; Fig. 3.1). We consider that such an important load variation would make the symbiont much less able, if not completely unable, to provide a significant amount of metabolites to its arthropod host (Fig. S1A and B). We argue that even if the Lab strain is not fully devoid of symbionts, the 5 or 6 orders of magnitude difference in *M. mitochondrii* density (*gyrB*/*cal* ratio=1e+01 versus 1e-5) should be sufficient to be responsible for a high portion of the differential gene expression between the two strains.

3.4.2 Generation of the RNA-seq dataset, *de novo* assembly and annotation

A total of 1,912,799,172 paired reads were obtained from the sequencing of the 35 librairies. After quality control filtering, the final data set contained 1,769,674,324 trimmed reads, with a mean number per library of 25,281,062 reads (range 14,862,878 - 42,759,000) (Table S1). The meta-assembly spanned 65.8 megabases and resulted in 36,499 contigs,

with an N50 of 2,738 bases and 30,207 Trinity 'genes' (which is the metrics retained by RSEM and edgeR-limma). The last metrics is comparable to the gene number of *I. scapularis* genome (Gulia-Nuss et al., 2016) despite being slightly larger, probably indicating some redundancy remaining even after transcriptome compaction. TransDecorder enabled the prediction of 47,822 peptides (Table S2). The Trinotate pipeline allowed the annotation with BLASTX of 83.89% of transcripts using the UniRef90 database, 71.40% (26,417/36,999) using the *I. scapularis* proteins database, and 51.51% using the Swissprot database (File SF1). Of note, even if not used by the subsequent post-hoc enrichment analyses (see result section 4 and 5), this last BLAST allowed to retrieve 50.56% of transcripts for GO terms and 46.16% for KEGG annotations (encompassing the same taxa as Swissprot, e.g. not *I. scapularis*). Using InterProScan, 12,085 GO annotations were retrieved among the 30,207 Trinity genes (40.0%). Other metrics can be found in Table 3.1 and Table S2. To assess transcriptome completeness, BUSCO genes were searched for the Arthropoda and Arachnida databases. With 93.7% and 91.9% completeness respectively, the assembly can thus be considered of excellent quality. Raw Illumina NovaSeq data have been deposited in GenBank under BioProject PRJNA662253.

3.4.3 Exploratory analyses comparing the 35 libraries

Similarities and differences among the 35 libraries were explored thanks to MDS plots showing relationships between libraries (Fig. 3.2) but also boxplots plotting the average expression profile of each library (Fig. S2) .The MDS plot using dimension 1 and 2 shows that the libraries are well clustered by organ and engorgement status (Fig. 3.2A). The eccentric location of the plot corresponding to salivary glands at D4 (around -6 in dimension 1) highlights the fact that they correspond to the most distinct librairies. Indeed, while the first axis is more related to the organ, the second axis corresponds more to the engorgement status. The clusterization by time (engorgement level) appears evident when plotting the dimensions 2 and 3 (Fig. 3.2B). Finally, the variability between the WT and Lab strains are more easily distinguished by the 4th and 5th dimensions (Fig. 3.2C), highlighting that the gene expression differences between those two sets of libraries are more difficult to identify.

Concerning the ovary libraries, the first axis explains the engorgement status while the second axis explains the strains (Fig 3.3). For this tissue, it should be noted that the strain variability effect seems to be more important in the D0 than in the D4 ticks. This pattern was not seen in Fig. 3.2A, illustrating that it can only be seen when analyzing

Metrics		Number
Number of contigs		36,499
Number of TRINITY genes		30,207
Percent GC (%)		51.58
Contig N50		2,738
Average contig length		1,802
Total assembled bases		65,769,310
BUSCO		
Arthropoda (%)		1,013 (100)
	Complete	949 (93.7)
	Single-copy	701 (69.2)
	Duplicated	248 (24.5)
	Fragmented	7 (0.7)
	Missing	57 (5.6)
Arachnida (%)		2,934 (100)
	Complete	2,697 (91.9)
	Single-copy	1,980 (67.5)
	Duplicated	717 (24.4)
	Fragmented	4 (0.1)
	Missing	233 (8)

Table 3.1 – **Assembly metrics for the transcriptome used in the differential expression analysis**

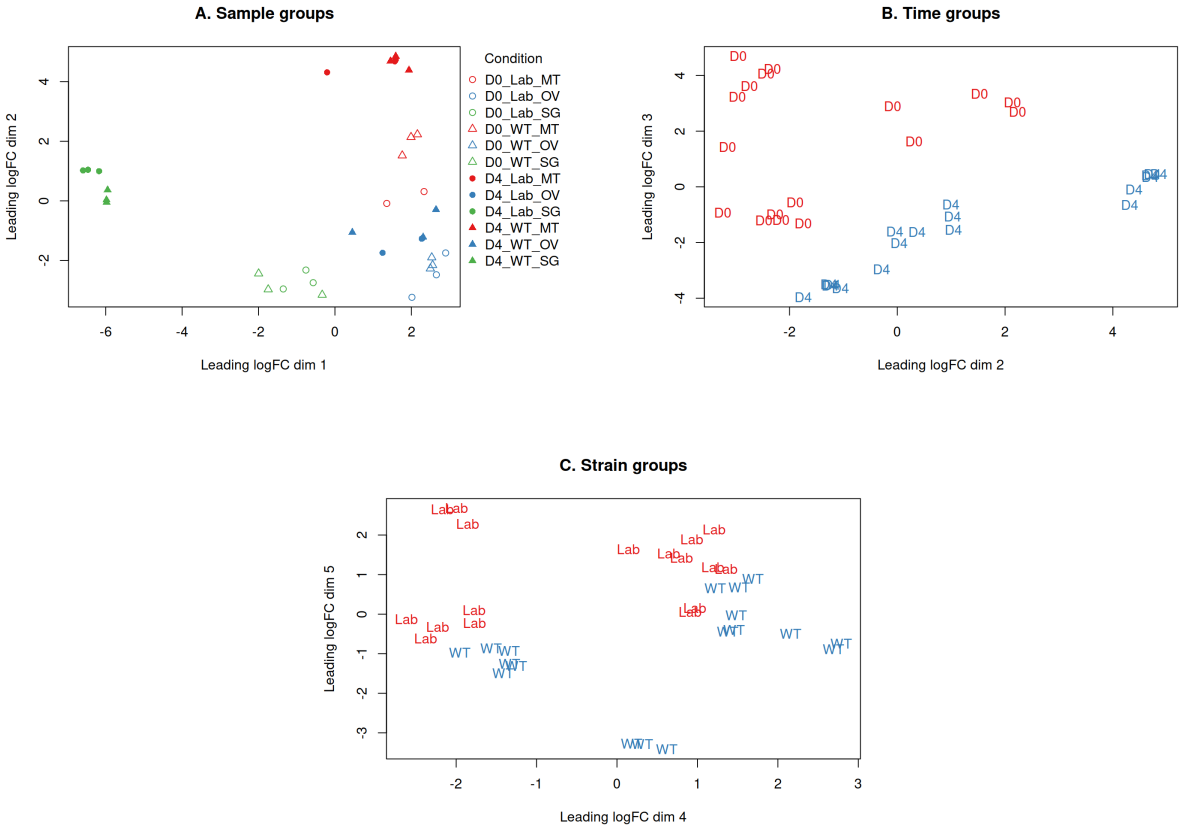

Figure 3.2 – **MDS plots of the libraries.**
A: for all sample groups in dimensions 1 and 2, B: for all time groups in dimensions 2 and 3 and C: for all strain groups in dimensions 4 and 5.
OV: ovary, MT: Malpighian tubules, SG: salivary glands, D4: 4 days after the deposit on the host, D0: before the deposit on the host, unengorged stage. Lab: Neuchâtel laboratory strain, WT: individuals collected from the wild

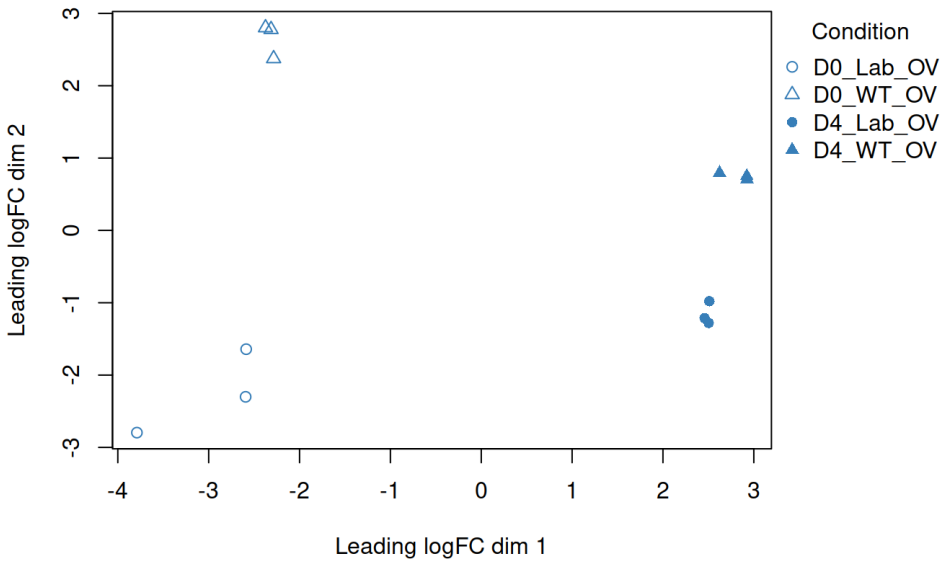

Figure 3.3 – **MDS plot for the ovary libraries.**
OV: ovary, D4: 4 days after the deposit on the host, D0: before the deposit on the host, unengorged stage.

each organ independently. This trend is not clear for the Malpighian tubules (Fig. S3A) with one of the two D0_Lab_MT libraries being an outlier. Note that the 3rd library had failed for amplification (see Materials and Methods), showing that the libraries obtained from this organ at D0, before their increase in size following the beginning of the blood meal, are based on limited amount of mRNA. This could possibly favor the presence of contaminating reads from other tissues, not fully separated during dissection. Finally, the strain effect is more pronounced at D4 than at D0 for the salivary glands (Fig. S3B).

The differential expression results presented below involve multilayered comparison. As seen in Fig. 3.2 and 3.3, the feeding status is more explicit than the symbiotic status. Therefore, the following section has been structured as such: (1) comparison of partially-fed *versus* unfed ticks for the (a) ovary, (b) Malpighian tubules and (c) salivary glands, followed by (2) comparison of ticks harbouring or not *M. mitochondrii* endosymbiont for the (a) the ovary, (b) Malpighian tubules and (c) salivary glands.

Differential expression analyses of tick tissues are challenging to interpret, notably because (i) they involve tens of thousands of genes, (ii) those genes are poorly annotated and their biological functions are most of the time poorly characterized and (iii) biological processes and metabolism are partly shared between the different organs. We thus decided to start the analysis of differential gene expression by comparing the conditions (partially-fed vs fed) where differences appeared more evident (Fig. 3.2A and 3.2B).

3.4.4 Differential Expression results comparing partially-fed *versus* unfed ticks

Overall analysis

First, differential expression analyses were performed for each tissue comparing D4 vs D0 ticks in (i) WT only or (ii) Lab only (Fig. 3.4). A third comparison dubbed 'global' encompasses both WT and Lab libraries together, as 6 replicates for each time point (except for the D0_MT condition; Table 3.2), the focus being on the investigation of the feeding effect independently of *I. ricinus* strains. All references to upregulated genes refer to upregulated in the D4 time point vs D0 time point.

Among all the upregulated genes in a given condition (ranging from 5,561 to 7,737;

Table 3.2 – **Number of up- or downregulated genes in all tissues for the D4 vs D0 comparison.**
D0 is used as the reference, e.g. upregulated means upregulated in D4 relatively to D0. OV: ovary, MT: Malpighian tubules, SG: Salivary glands, WT: WT strain ticks, Lab: Lab strain ticks, global: regardless of the strain, Up: number of upregulated genes, Not DE: number of not differentially expressed genes, Down: number of downregulated genes.

	OV WT	OV Lab	OV global	MT WT	MT Lab	MT global	SG WT	SG Lab	SG global
Up	6,951	7,509	7,344	5,561	7,560	6,717	6,948	7,737	7,405
Not DE	19,119	18,530	18,713	19,938	17,284	18,310	17,839	16,158	16,798
Down	2,825	2,856	2,838	3,396	4,051	3,868	4,108	5,000	4,692

Table 3.2) around one third (2,190) were common in the three organs and in both tick strains (first bar of the Fig. 3.4 histogram). The same pattern is observed for the downregulated genes (1,418 in a range of 2,825-5,000; third bar of the Fig. 3.4 histogram). This illustrates that a large number of genes (3,608) were DE independently of the organ and the strain. The second (1526) and fifth (1008) sets of DE genes corresponded respectively to genes downregulated and upregulated solely in the salivary gland of both tick strains, illustrating that this specific organ exhibits a more contrasted DE gene set, compared to the other organs (Fig. 3.4).

Ovary

Gene counting After filtration of the poorly expressed genes, the number of unbiased genes was 28,895. In the ovary, for the D4 vs D0 comparison and in the WT strain, 6,951 genes were found to be upregulated, 2,825 were downregulated, while 19,119 genes were found to not be DE. For the Lab strain, 7,509 genes were upregulated and 2,856 were downregulated, while 18,530 were found to be non significantly differentially expressed. For the "global" comparison (whether WT or Lab strain considered), 7,344 genes were upregulated and 2,838 were downregulated, while 18,713 were found to not be DE (Table 3.2).

Clusterization A heatmap of D0 and D4 DE genes in the ovary (Fig 3.5) allowed the visualisation of the expression pattern of all the genes. There was a clear clusterization for D4 libraries, independently of the strain (WT or Lab). The three D0_Lab_OV libraries clustered together according to the cladogram but the branch length corresponding to the D0_Lab_OV_3 library was greater than the other internal branches suggesting that this library exhibited an atypical gene expression pattern.

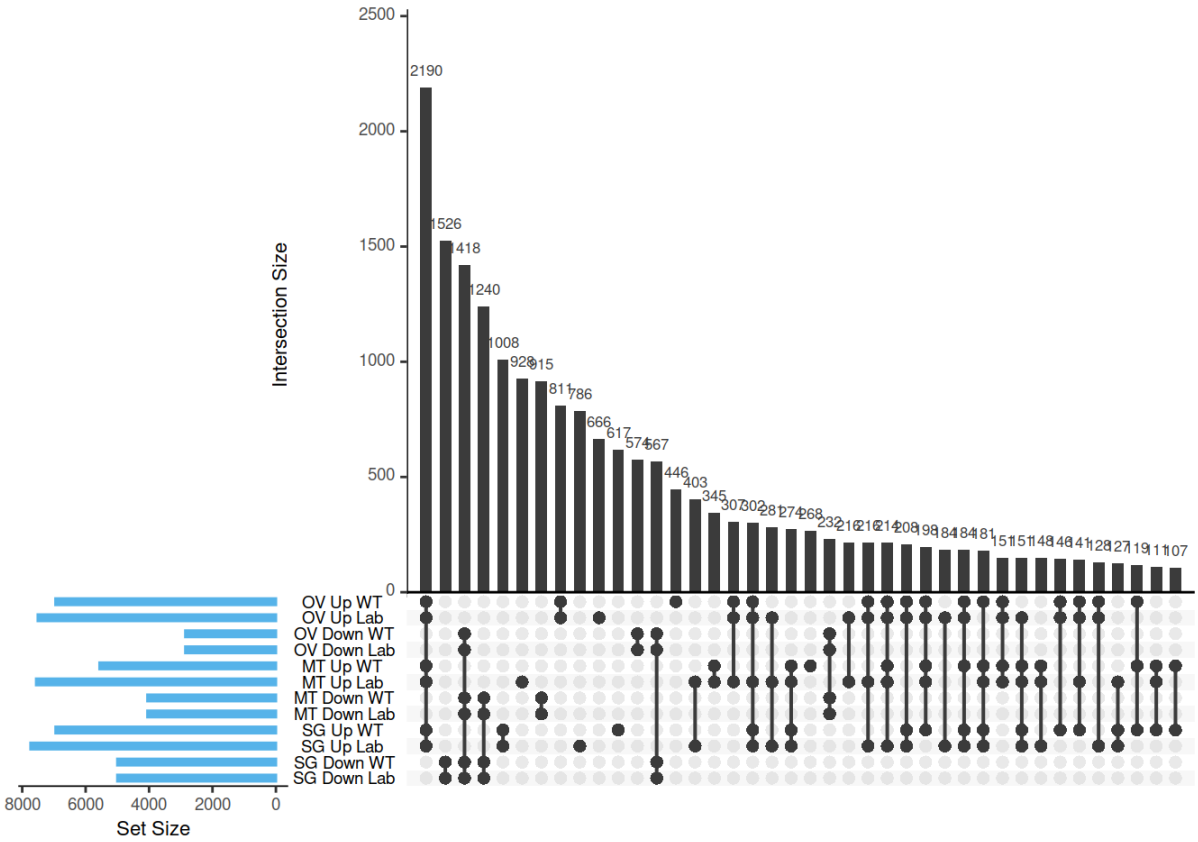

Figure 3.4 – **Upset plot of differentially expressed genes between D4 and D0 in one or multiple conditions, whether they are upregulated (Up) or downregulated (Down), in the different organs (OV, SG, MT) or strains (WT, Lab).** A black dot indicates the presence of DE genes for the condition named on the left. Dots linked by lines represent DE genes in multiple conditions. Vertical bars above the dots stand for the number of DE genes in the condition marked with a dot. Horizontal blue bars on the lower left indicate how many genes are DE in each condition. OV: ovary, MT: Malpighian tubules, SG: salivary glands, Up: Upregulated, Down: Downregulated, WT: Wild-type strain ticks, Lab: Lab strain tick

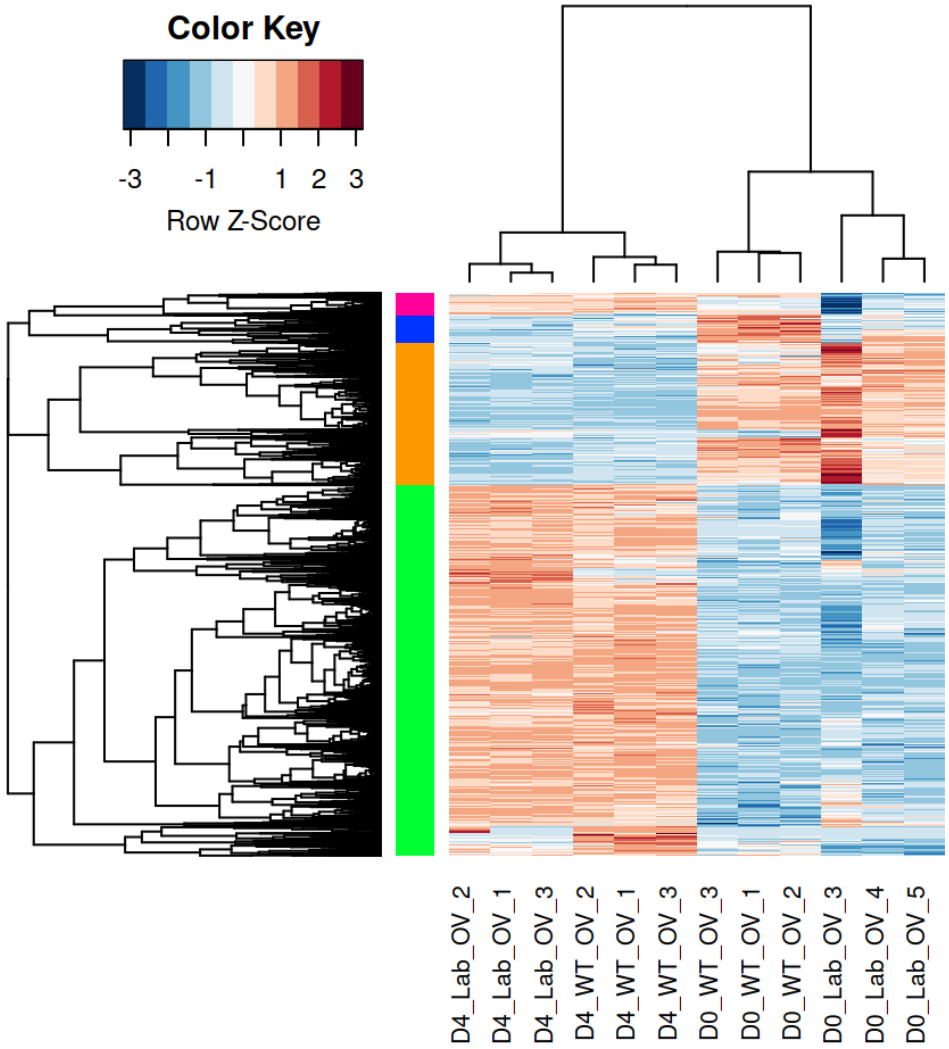

Figure 3.5 – **Heatmap based on the log count-per-million data of the ovary at D0 and D4.**
Low expression values tend towards dark blue while high expression values tend towards dark red. Hierarchical clustering (threshold set at k = 4) of genes is shown by the colours on the left side (blue, orange, pink and green).

Gene ontology and gene set analyses

Upregulated genes Gene ontology enrichment was run on the DE genes using topGO (TableSupporting information) and goseq (Table 3.5), which gave overall similar results. The following sections take advantage of topGO results (Table 3.4; Table 3.5 can be viewed online, see Supporting information section). It should be noted that mentions such as "common when considering either WT or Lab libraries" or "shared between the WT and Lab comparisons" are statistically different from the otherwise indicated "global comparison": the former relies on the comparison of common enriched terms in the independant WT or Lab DE gene calculation (three replicates each, beside the two replicates for the MT_Lab_OV condition), the latter relies on the overall comparison of the six libraries for each D4 or D0 time point.

Regarding the upregulated genes (at D4 compared to D0), most top 10 terms are common when considering either the WT or Lab libraries, e.g. 8 out 10 biological process (BP) terms such as "protein phosphorylation", "signal transduction", "regulation of transcription, DNA-templated", "intracellular signal transduction", "cytoskeleton organization", "DNA integration", "cell adhesion" and "synapse organization" are shared. The term "protein phosphorylation" which is the most enriched term in all D4 vs D0 comparisons (regardless the strain or the organ) include notably a wide variety of kinases more or less well annotated and characterized, for instance in the ovary for the WT strain, including tyrosine kinases, casein kinases, serine/threonine kinases, microtubule associated serine/threonine kinases, Rho-associated kinases, ribosomal protein S6 kinases, but also a tumor necrosis factor (TNF) receptor-associated factor (TRAF), some TRAF 6-like or a vascular endothelial growth factor receptor.

For the global D4 vs D0 comparison (Table 3.4), the enriched terms depict the same trend. The term "lipid transport" which could be important for the oogenesis process is in the 8th position of this list, whereas it was 13th and 23th in the WT and Lab lists, respectively. Among the 50 annotated-as-such genes in the whole data set, 22 were up-regulated. *Ixodes scapularis* BLASTX description of these DE genes was notably associated with several hemelipoglyco-carrier proteins and to several (phospholipid-transporting) ATPases (Table SF1). Hemelipoglyco-carrier protein sequences are close to the well-known vitellogenins (involved in lipid transport; Mitchell et al., 2019). Two vitellogenins are described

in the *I. scapularis* genome. Of note, direct orthologs of the two described vitellogenin proteins in the *I. scapularis* genome (Vg1 ISCW013727 and Vg2 ISCW021228, according to VectorBase) were not found in the *de novo* contigs generated in this study, only more distant BLAST results more related to hemelipoglyco-carrier proteins. Nevertheless, we found them in other *I. ricinus* published transcriptome in TSA (e.g. GADI — `https://www.ncbi.nlm.nih.gov/Traces/wgs/GADI01`). However, this lack of vitellogenin transcripts in the ovary is not surprising given that vitellogenins are typically expressed in fully fed ticks, and not in unfed or partially-fed females, and preferentially in the fat bodies rather than the ovary where vitellogenins are transported and transformed in their functional vitellin form, providing nutrients for the developing embryos (Donohue et al., 2008; Donohue et al., 2009; Khalil et al., 2011; Smith and Kaufman, 2014).

Some BP terms of relevance are found to be enriched in WT and not in Lab, and vice versa, notably: "translation", "mitochondrial calcium ion homeostasis", interestingly "embryonic morphogenesis", "lipoprotein metabolic process" and "mitotic spindle organization" (for the WT strain); "regulation of cellular component biogenesis" , "ion transport", "developmental process", "regulation of mitotic cell cycle", "exocytosis", "intracellular transport", "regulation of the developmental process" (for the Lab strain).

Regarding the top 10 of the molecular function (MF) topGO enrichment (Table 3.4), functions such as "protein binding", "protein serine/threonine kinase activity", "ATP binding", "calcium ion binding", "actin filament binding", "microtubule binding", "guanyl-nucleotide exchange factor activity" and "protein kinase activity" are shared between the WT and Lab condition, and are also found in the "global" comparison. It is worth to be noted that the term "lipid transporter activity", more or less similar to the BP term "lipid transport" regarding the genes that compose this term, is ranked 10th in WT, 12th in Lab and 12th in global (i.e. without considering any WT or Lab difference).

Regarding the cellular component (CC) terms (Table 3.4), most terms are shared between the WT and Lab comparisons, such as "cell-cell junction", "myosin complex", "cytoskeleton", "plasma membrane", "extracellular space", "spindle", "intracellular organelle" or "microtubule cytoskeleton". The terms related to the cytoskeleton complex support the fact that the ovarian cells are evolving upon engorgement.

For the goseq enrichment method (Table 3.5, the top 10 terms of all ontologies taken together are shown in the SF3 supplementary file. Terms such as "protein phosphorylation" (BP), "protein binding" (MF), "ATP binding" (MF), "signal transduction" (BP), "regulation of transcription, DNA-templated" (BP) are shared between topGO and goseq, strengthening the previously mentioned idea of a metabolism switch towards the development and activity of the ovarian tissue.

Another approach to consider is gene set analysis enrichments, which have been done using romer (file SF2) and camera (file SF3). Romer and camera confirm that some of the functions based on gene sets constituted by GO terms are indeed significantly enriched globally, such as "actin cytoskeleton organization", "cytoskeleton organization", "cell adhesion", "(intracellular) signal transduction" or "lipid transport". However the large set "protein phosphorylation" is only significantly enriched according to the camera method (file SF3) and not with romer (file SF2).

Gene set enrichment approach was also conducted to create custom gene sets, e.g. after text mining the pattern "vitellogenin|Hemelipoglyco" (for vitellogenin receptors and hemelipoglyco-protein related genes) in the BLASTX description results of the different databases. By this approach, a gene set of 14 genes was created and the statistical analysis revealed that the set was significantly enriched (whether using romer or camera softwares, FDR p-values of 3.8e-03 and 2e-04 respectively) towards the partially-fed ticks (global comparison; Fig. 3.6).

Globally, independently of the strain and regarding the terms that are upregulated, the top 10 BP, MF, CC of topGO (Table 3.4), the top 10 terms of goseq (all ontologies taken together; Table 3.5), the results of romer (file SF2) and camera (file SF3) show deep cellular and biological modifications of the ovary, especially regarding cytoskeleton (including microtubules and actin), which would be congruent with what we could expect for a partially-engorgement stage and the metabolic changes occurring in the first days of blood feeding. In particular, after having been produced in other organs such as fat bodies, the integration/fusion of lipid droplets into ovary cells may involve the genes corresponding to those GO terms.

Downregulated genes It should be noted that not all oocytes are fully mature at D4, as oocyte maturation is a continuous process occurring throughout the whole en-

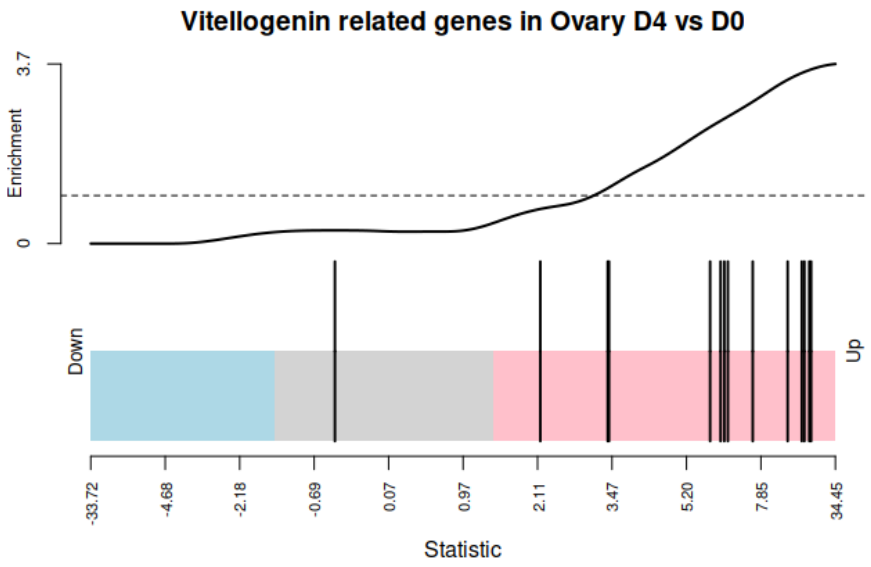

Figure 3.6 – **Barcode plot of vitellogenin/hemelipoglyco-carrier protein related genes in the global D4 vs D0 comparison**
A black bar represents a gene. The statistic chosen is the moderated t-statistic calculated by the eBayes limma-trend function. The moderated t-statistic (X axis) is the ratio of the M-value (log2-fold change) to its standard error. The background color in rose and blue correspond to genes that are up- and downregulated respectively, while the grey color correspond to non DE genes

gorgement (Sonenshine and Roe, 2013). Enriched topGO BP terms such as "iron-sulfur cluster assembly" and "cofactor metabolic process" are shared between the WT and Lab comparisons (Table 3.4). Interestingly, those terms include genes annotated (according to the UniRef90 database) as frataxin (file SF1). Frataxin is an essential mitochondrial protein believed to be an iron chaperone, binds to ferrochelatase and other components of the iron-sulfur cluster machinery. It could thus be implicated in heme metabolism (Adinolfi et al., 2009). This assumption is reinforced by the enrichment (FDR p-values <0.05) of the terms "iron-sulfur cluster assembly" (BP), "4 iron, 4 sulfur cluster binding" (MF), "iron ion binding" (MF), "heme binding" (MF), "response to metal ion" (BP), "iron-sulfur cluster binding" (MF) and "protein maturation by [4Fe-4S] cluster transfer" (BP) by a camera enrichment test (file SF3).

Regarding BP terms, around half of those terms are shared when considering the WT and Lab comparisons, although 24 terms are unique for each of both comparisons (Table 3.4), e.g. "mitochondrial transcription", "oxidation-reduction process", "rRNA processing" "regulation of cellular process" or "positive regulation of intracellular signal transduction" are unique for the WT; "DNA-templated transcription, initiation", "RNA metabolic process" or "transcription, DNA-templated" are unique for the Lab.

The same trend appears to be evident for the MF terms, terms such as "threonine-type endopeptidase activity", "nucleic acid binding", "iron-sulfur cluster binding" or "methyltransferase activity" are shared between the WT and Lab comparison (22 in total), but 18 are exclusive to the WT (such as "iron ion binding", "heme binding" or "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen") while 23 are exclusive to the Lab (such as "transcription coregulator activity", "magnesium ion binding" or "DNA binding"). The results described in this section could imply that the difference between the strains at the unfed stage is more marked compared to the partially-fed stage.

Malpighian tubules

Gene counting In the Malpighian tubules (MT), regarding the D4 vs D0 comparison in the WT condition, 5,561 genes were found to be upregulated, 3,396 were downregulated, while 19,938 genes were found to be non significantly differentially expressed. For the Lab condition, 7,560 genes were upregulated and 4,051 were downregulated, while

17,284 were found to be non significantly differentially expressed. For the global comparison (all MT libraries considered, whether WT or Lab condition considered), 6,717 genes were upregulated and 3,868 were downregulated, while 18,310 were found to not be DE (Table 3.2).

Clusterization A heatmap of D0 and D4 DE genes in the Malpighian tubules allows the visualisation of the expression pattern of all the genes. While thesix6 D0_OV libraries grouped together in the cladogram — and they also cluster according to strains — (Fig. 3.5), it appears to not be the case for the 2 D0_Lab_MT libraries (with D0_Lab_MT_4 being the sister group to the 4 remaining D0_WT and D0_Lab_MT_5 libraries (Fig. 3.7)

Gene ontology and gene set analyses

Upregulated genes (enriched in partially-fed ticks) When considering the global comparison, the topGO enrichment (Table 3.4) indicates that 34 enriched BP terms are shared between the ovary and the Malpighian tubules for the partially-fed ticks, in particular terms with a very small p-values such as “protein phosphorylation”, “regulation of transcription, DNA-templated”, “(intracellular) signal transduction”, “cell adhesion”, “DNA integration”, “(actin or microtubule) cytoskeleton organization”, “microtubule-based movement” or “developmental process”. Miscellaneous binding or activity molecular functions are also common between the ovary and Malpighian tubules (Table 3.4), such as “protein binding”, “ATP binding”, “microtubule binding”, “protein serine/threonine kinase activity”, “calcium ion binding”, “actin (filament) binding” or “protein kinase activity” (36 MF terms common in total).

Still regarding the global comparison, some BP and MF terms following engorgement in the ovary and Malpighian tubules are therefore common, notably regarding the cytoskeleton reshapement, the transcription or the cellular transduction processes. Yet, all the processes are obviously not common, we found 26 enriched BP terms (e.g. “lipid transport”) and 20 MF terms (e.g. “DNA-binding transcription factor activity” or “lipid binding”) unique to the ovary; while we found 28 enriched BP terms (e.g. “translation” or “regulation of cellular component biogenesis”) and 18 MF terms (e.g. “structural constituent of ribosome” or “histone-lysine N-methyltransferase activity”) unique to the Malpighian

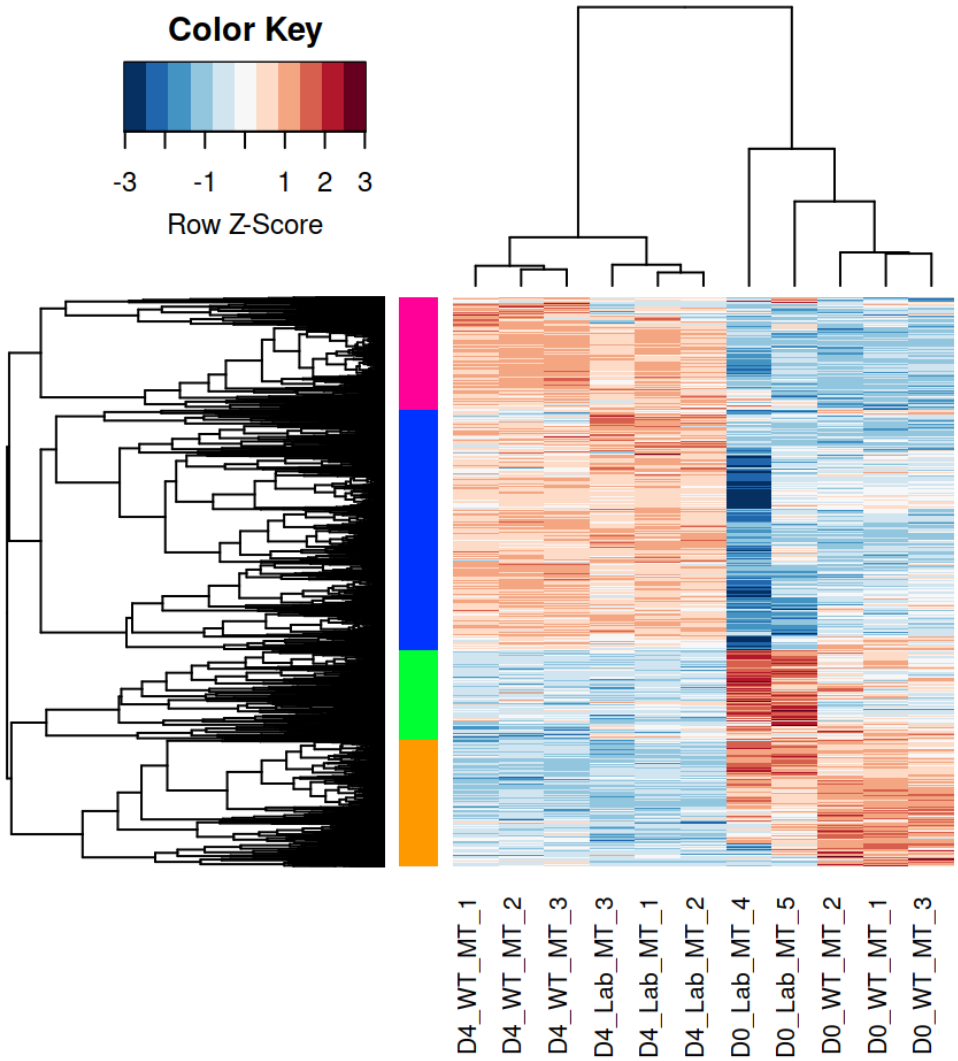

Figure 3.7 – **Heatmap based on the log count-per-million data of the Malpighian tubules at D0 and D4.**
Low expression values tend towards dark blue while high expression values tend towards dark red. Hierarchical clustering (threshold set at k = 4) of genes is shown by the colours on the left side (blue, orange, pink and green).

tubules (Table 3.4).

Regarding the terms common to both strains (global comparison; Table 3.4), there are 30 BP terms (e.g. "protein phosphorylation", "translation" "(small GTPase mediated/intracellular) signal transduction", "(regulation of) microtubule cytoskeleton organization" etc.) and 39 MF terms (e.g. "ATP binding", "microtubule binding", "protein binding", "structural constituent of cytoskeleton"). Among the exclusive terms to the WT strain, we can find the 31 BP terms "microtubule-based process", "regulation of cell cycle" or "mitotic chromosome condensation" and the (12) MF terms like "structural constituent of cuticle", "ATPase activity" or "calmodulin binding". Regarding the terms unique to the Lab strain, there are the 19 BP terms "ATP synthesis coupled proton transport", "regulation of signal transduction" or "protein-containing complex assembly" and the 13 MF terms "ion channel activity", "helicase activity" or "RNA polymerase II transcription regulatory region sequence-specific DNA binding".

The romer (file SF2) and specifically camera (file SF3) analyses confirm that overall terms related to cytoskeleton, transduction or translation are significantly enriched for both strains (global comparison, enriched in partially-engorged ticks).

Downregulated genes The topGO analysis (Table 3.4) for the terms differentially expressed in unfed ticks show that there are 8 BP terms shared when considering the WT and Lab comparisons (in particular the term "oxidation-reduction process") and 14 MF terms in common (e.g. "iron ion binding", "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen", "heme binding", "sulfotransferase activity" or "methyltransferase activity"). Among the DE genes with the most important LFCs and the smallest p-values, several genes are annotated as sulfotransferases.

However, it seems that there is a greater diversity between the two strains, with 22 BP terms (e.g. "transmembrane transport" or "alpha-amino acid metabolic process") and 20 MF terms (e.g. "transmembrane transporter activity", "growth factor activity", "chitin binding" or "GTP binding") unique to the WT strain, and for the Lab strain, with 34 unique BP terms (e.g. "positive regulation of JNK cascade", "activation of NF-kappaB-inducing kinase activity", "iron-sulfur cluster assembly", "RNA metabolic process" or

"cilium assembly") and 17 MF terms (e.g. "cytokine receptor binding" or "protein heterodimerization activity").

The romer (file SF2) and camera (file SF3) results confirm overall the results found by topGO (Table 3.4) and goseq(Table 3.5), with terms such as "heme binding", "iron ion binding", "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen", "oxidation-reduction process" or "sulfotransferase activity" being in common between the two strains, but also curiously terms such as "activation of NF-kappaB-inducing kinase activity" and "positive regulation of JNK cascade" being significantly enriched only in the Lab strain.

Salivary glands

Gene counting In the salivary glands, regarding the D4 vs D0 comparison in the WT condition, 6,948 genes were found to be upregulated, 4,108 were downregulated, while 17,839 genes were found to be non significantly differentially expressed. For the Lab condition, 7,737 genes were upregulated and 5,000 were downregulated, while 16,158 were found to be non significantly differentially expressed. For the global comparison (whether WT or Lab condition considered), 7,405 genes were upregulated and 4,692 were downregulated, while 16,798 were found to be non significantly differentially expressed (Table 3.2).

Clusterization A heatmap of D0 and D4 DE genes in the salivary glands allows the visualisation of the expression pattern of all the genes, with the expression of the DE genes clustering according to time first, then strain (Fig. 3.8).

Gene ontology and gene set analyses

Upregulated genes Regarding the partially-fed ticks, the topGO enrichment (Table 3.4) emphasizes 28 BP terms (as mentioned above for the ovary and Malpighan tubules, terms such as "protein phosphorylation", "(intracellular) signal transduction", "cell adhesion", "(actin) cytoskeleton organization", but also specific to the salivary glands, e.g. "mitigation of host defenses by symbiont", "proteolysis", "nucleotide catabolic process") and 44 MF terms (e.g. "amine binding", "structural constituent of cuticle", "protein serine/threonine kinase activity", "serine-type endopeptidase inhibitor activity" or "metal-

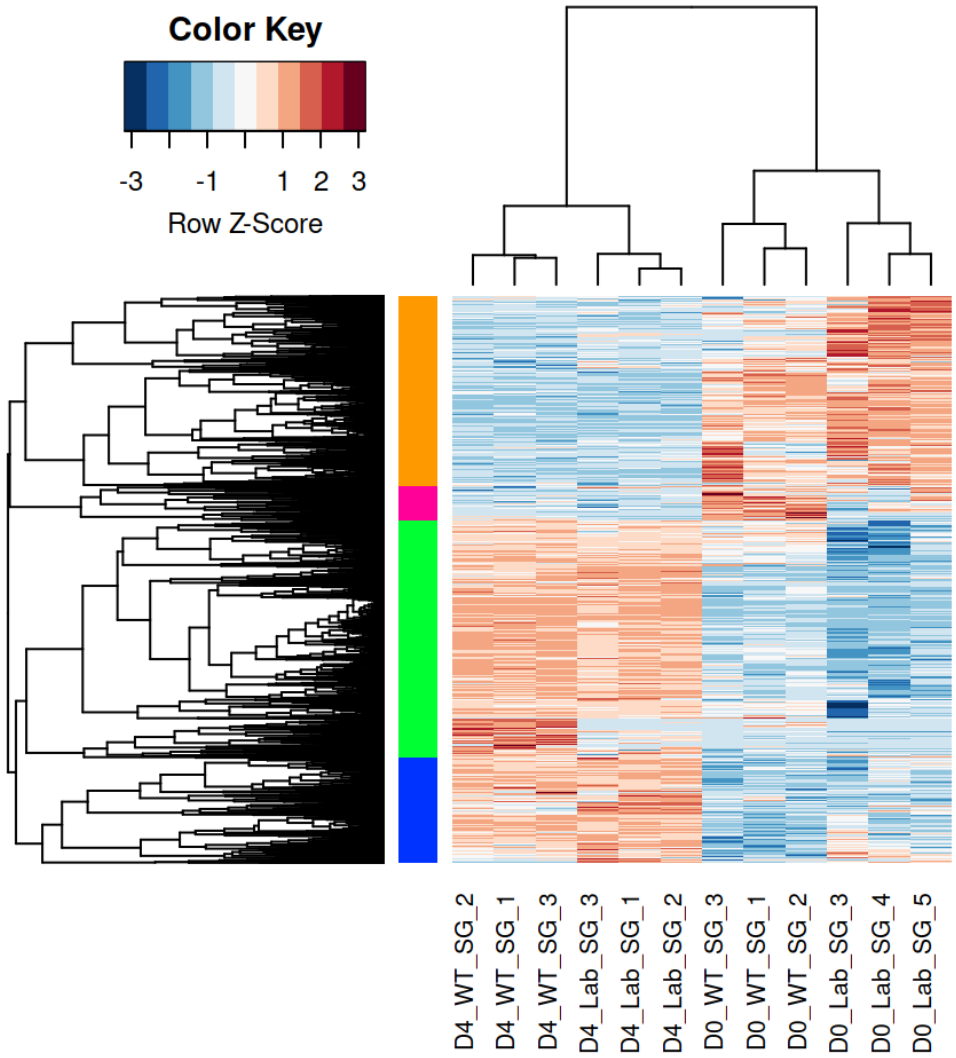

Figure 3.8 – **Heatmap based on the log count-per-million data of the salivary glands at D0 and D4.**
Low expression values tend towards dark blue while high expression values tend towards dark red. Hierarchical clustering (threshold set at k = 4) of genes is shown by the colours on the left side (blue, orange, pink and green).

loendopeptidase activity") common to the WT and Lab strain comparisons.

Regarding the terms unique to each strain, the WT strain denotes 10 BP terms (e.g. "translation" or "secretion by cell") and 13 MF terms (e.g. "metallopeptidase activity", "serine-type endopeptidase activity", "structural constituent of ribosome" or "endopeptidase inhibitor activity") while for the Lab strain there are 32 BP terms (e.g. "protein ADP-ribosylation", "regulation of cellular component biogenesis", "protein-containing complex assembly" or "transmembrane transport") and 18 MF terms ("hydrolase activity, acting on ester bonds", "ATPase activity", "GTPase activator activity" or "ATPase-coupled transmembrane transporter activity") (Table 3.4).

The camera test (file SF3) indicates that terms such as "actin cytoskeleton organization", "cell adhesion", "cytoskeleton", "lipid transporter activity" or "serine-type endopeptidase inhibitor activity" (the latter only for the WT libraries) are globally enriched for the partially-fed ticks (global comparison).

Downregulated genes As for the unfed ticks, we tally 13 common BP terms (e.g. "DNA replication", "oxidation-reduction process", "positive regulation of JNK cascade" or "activation of NF-kappaB-inducing kinase activity") and 12 common MF terms (e.g. "iron ion binding", "heme binding" or "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen") between the WT and Lab strains (Table 3.4).

Again, for this organ this time, a greater diversity between the strains is apparent. We count 19 BP terms (e.g. "mitigation of host defenses by symbiont", "rRNA processing", "response to external stimulus" or "RNA splicing") and 11 MF terms (e.g. "amine binding", "transcription coregulator activity", "RNA binding", "hydrolase activity, acting on glycosyl bonds" or "GTP binding) only significantly enriched for the WT libraries. As for the Lab strain, we find 29 BP terms (e.g. "iron-sulfur cluster assembly", "ion transmembrane transport", "RNA metabolic process", "actin filament organization" or "programmed cell death") and 25 MF terms (e.g. "extracellular ligand-gated ion channel activity", "methyltransferase activity", "zinc ion binding", "oxidoreductase activity", "iron-sulfur cluster binding" or "sulfotransferase activity") exclusively enriched in this strain (Table 3.4).

The camera results (file SF3) show that GO annotated genes such as "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen", "iron ion binding", "activation of NF-kappaB-inducing kinase activity", "positive regulation of JNK cascade" or "oxidation-reduction process" are enriched for the WT strain. As for the Lab strain, terms such as "nucleotide catabolic process", "intracellular signal transduction", "protein phosphorylation" or "signal transduction" are brought out.

3.4.5 Overall differential expression results for the WT vs Lab library comparison

Differential expression analyses were performed for each tissue, comparing WT and Lab ticks at D0 and D4 time points, without any "global" comparison, as the feeding status effect is largely stronger than the symbiotic status. In the following section, the focus is on the investigation of the effect of *M. mitochondrii* on *I. ricinus* gene expression. Therefore, all references to "upregulated" genes refer to the genes upregulated in the WT ticks compared to the Lab ticks (the reference being the Lab ticks, without *M. mitochondrii*).

Contrary to the same analysis conducted to compare D4 vs D0 libraries, no clear clustering (gathering of multiple conditions) is observed, either by organ or feeding status (Fig. 3.9), very few DE genes are common between all the condition, i.e. only 128 and 32 genes are respectively up- and down-regulated in all conditions. Quite the opposite, the singularly high relative number of DE genes in a solely given condition (for instance 1,811 up-regulated in the Malpighian tubules at D0, 1,347 up-regulated in the salivary glands at D0 or 1,343 down-regulated in the salivary glands at D0) pinpoints a likely specific tissue tropism depending on whether the WT or Lab strains. Please note that the total number of DE genes for each condition (blue horizontal bars) is also very reduced relative to the previous D4 vs D0 comparison (with 7 out of 12 sets below 1,500 genes; Fig. 3.4 and Table 3.2), as could be expected in two conditions (WT and Lab) differentiated by a single factor (presence of *M. mitochondrii*) rather than representing different organs or stages of development.

Ovary

Gene counting In the D4 condition, 521 genes were found to be upregulated, 405 were downregulated, while 27,969 genes were found to not be DE. In the D0 condition,

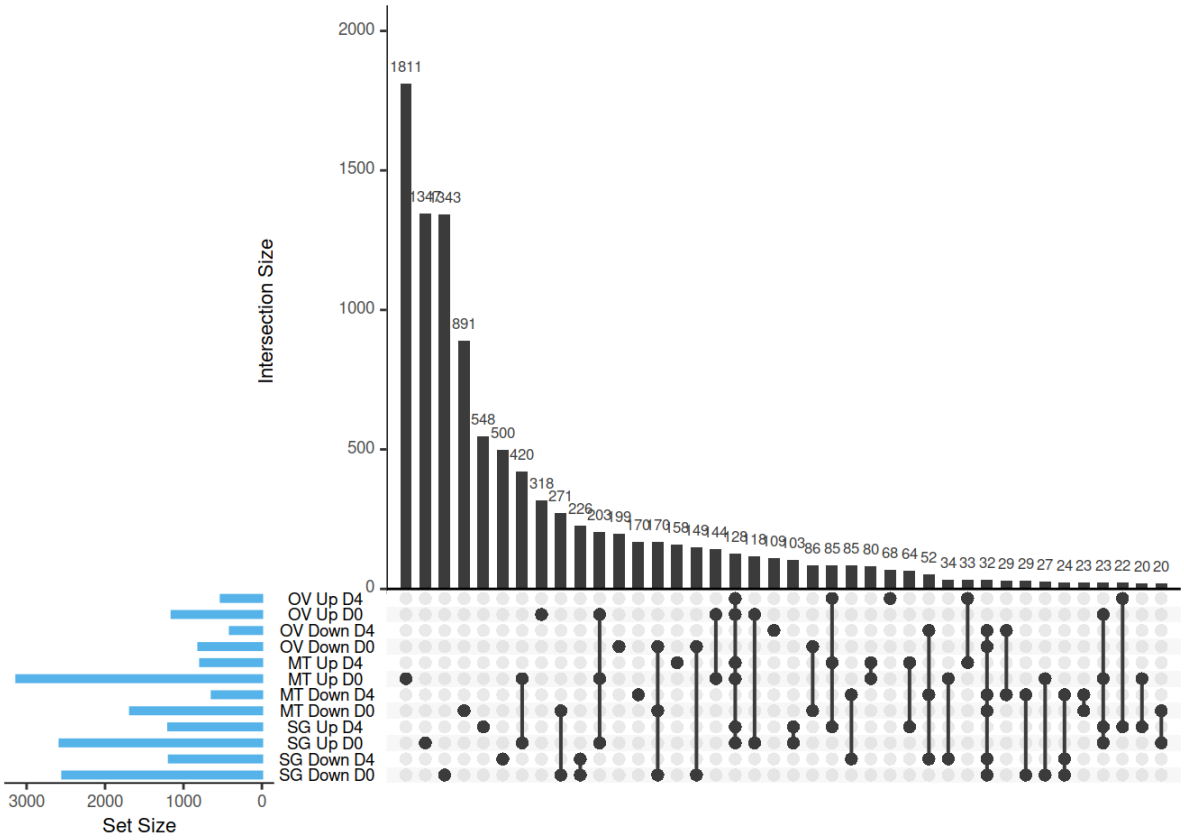

Figure 3.9 – **Upset plot of differentially expressed genes between WT and Lab in one or multiple conditions, whether they are upregulated or downregulated in the WT (Wild-type), in the different organs (OV, SG, MT) and per time points (D4, D0).** black dot indicates the presence of DE genes for the condition named on the left side. Dots linked by lines represent DE genes in multiple conditions. Vertical bars above the dots stand for the number of DE genes present in the condition marked with a dot. Horizontal blue bars on the lower left indicate how many genes are DE in each condition. OV: ovary, MT: Malpighian tubules, SG: salivary glands, Up: Upregulated, Down: Downregulated, D4: 4 days after the deposit on the host, D0: before the deposit on the host, unfed stage

Table 3.3 – **Number of up- or downregulated genes in all tissues for the WT vs Lab comparison.**
Lab is used as the reference, e.g. upregulated means upregulated in WT towards Lab. OV: ovary, MT: Malpighian tubules, SG: Salivary glands, D4: 4 days after the deposit on the host, D0: before the deposit on the host, unfed stage

	OV D4	OV D0	MT D4	MT D0	SG D4	SG D0
Up	521	1,148	784	3,128	1,191	2,576
Not DE	27,969	26,940	27,475	24,089	26,521	23,777
Down	405	807	636	1,678	1,183	2,542

more genes were differentially expressed, 1,148 genes were upregulated while 807 were downregulated, and 26,940 were found to not be DE (Table 3.3).

Clusterization A heatmap of D0 and D4 DE genes in the ovary allows the visualisation of the expression pattern of all the DE genes (Fig. 3.10A) with a clear clusterization of by strain then by feeding stage. An unsupervised clusterization by the Bi-Co-Pam method allows the identification of two clusters, the C0 cluster with 272 genes following the same trend (over expressed in both D0 and D4 WT and under expressed in both D0 and D4 Lab) and the C1 cluster with 282 genes following the opposite trend (Fig. 3.10B), meaning that those clusters are particularly interesting to assess a potential effect of the symbiont of the host gene expression.

Gene ontology and gene set analyses As above, the results from topGO (Table 3.6) and goseq (Table 3.7) are noticeably the same, although topGO seems to encompass more enriched terms (p-value < 0.05) than goseq. We will refering to the topGO results from now on.

Upregulated genes (overexpressed in WT ticks) More genes are differentially expressed in D0 than in D4 and logically lead to more significantly enriched terms in D0 than in D4. In addition, the same trend is depicted in the other organs (Table 3.3).
According to the topGO results (Table 3.6), no BP terms are common between the D0 and D4 comparisons.
The MF term "cysteine-type endopeptidase inhibitor activity" is common between the two time points, although 5 genes are DE in D0 while only 2 are DE in D4. Those two DE genes in D4 (D4_C_N_OV.trinity_TRINITY_DN5796_c0_g1 and D4_C_N_SG.trinity_TRINITY_DN6713

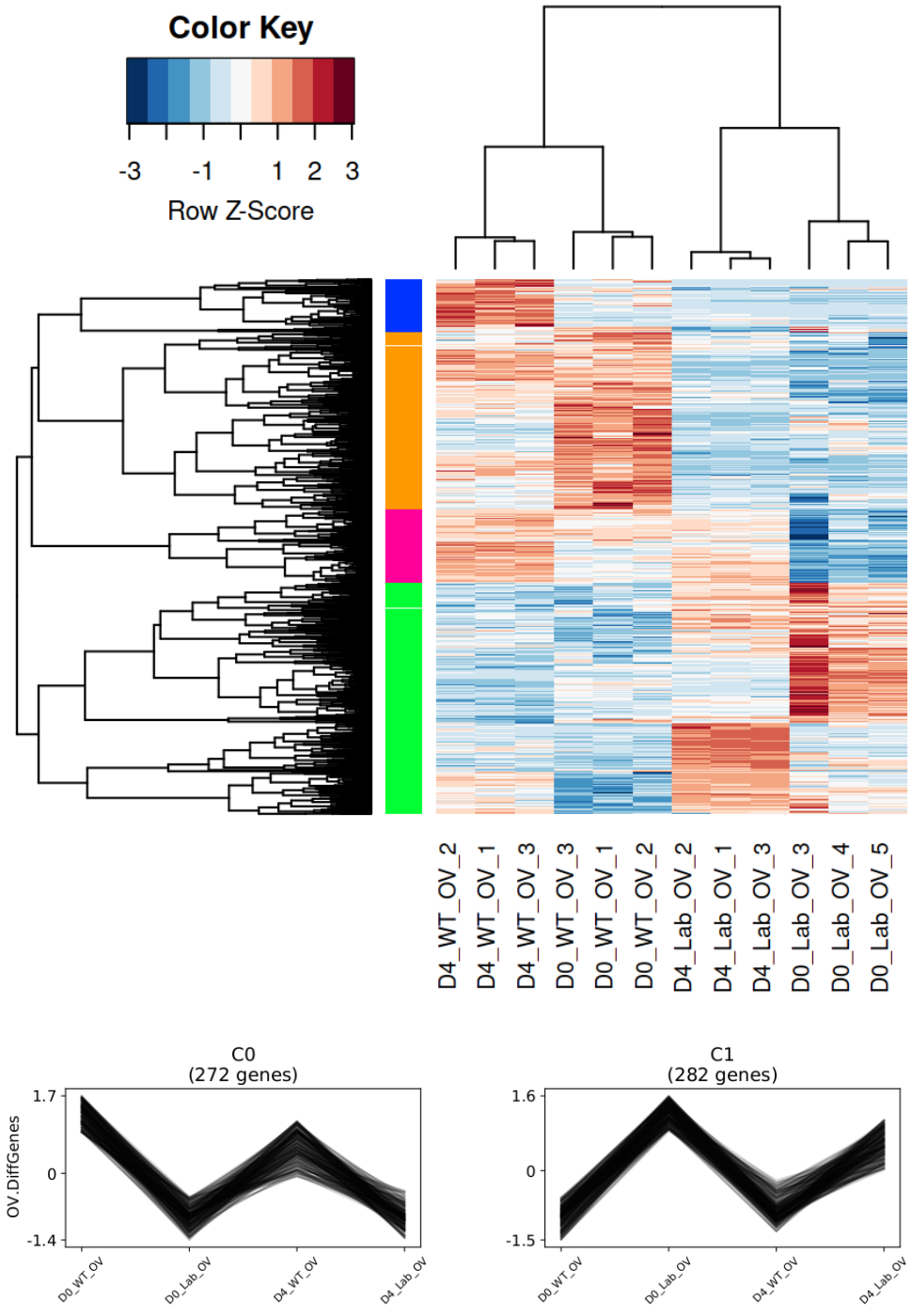

Figure 3.10 – **Expression trends of DE genes in the ovary for the WT vs Lab comparison.** A: heatmap based on the log-count-per-million data of the ovary at D0 and D4. Low expression values tend towards dark blue while high expression values tend towards dark red. Hierarchical clustering of genes is shown by the colours on the left side (blue, orange, pink and green). B: Gene co-expression profiles of the two gene clusters (see text for description), K-means clustering of the expression data of all sample types (x-axis) using the log-count-per-million metrics as input. The y-axis represents the normalised expression values.

are annotated as cystatin-L2 (according to UniRef90 BLAST description; file SF1), and among those two, only D4_C_N_OV.trinity_TRINITY_DN5796_c0_g1 is DE in D0. The other 4 are annotated as "secreted cystatin" or "putative salivary cystatin" (file SF1). Cystatins are cysteine protease inhibitors, the role of certain cystatins within ticks has been characterized and could trigger tick innate immunity as well as blood feeding abilities or embryogenesis (Schwarz et al., 2012).

The CC term "TIM22 mitochondrial import inner membrane insertion complex" is also enriched both in D0 and D4 (Table 3.6). The 2 DE genes in D4 corresponding to this term are annotated as "Mitochondrial import inner membrane translocase subunit Tim29" according to SwissProt ("Putative conserved protein with signal anchor" for UniRef90 and "uncharacterized protein" for *I. scapularis* genome; file SF1). Those two genes (D0_C_N_OV.trinity_TRINITY_DN6217_c0_g1 and D4_C_N_OV.trinity_TRINITY_DN30_ are also DE in D0, alongside D0_C_N_SG.trinity_TRINITY_DN27744_c0_g1 which is also annotated as Tim29 by SwissProt ("uncharacterized protein" according to UniRef90 and *I. scapularis* genome; file SF1).

Concerning the D0 enrichment results (Table 3.6), 16 unique enriched BP terms are tallied, including "oxidation-reduction process", "intermembrane lipid transfer", "mitochondrial cytochrome c oxidase assembly", "mitochondrial transport" and "mitochondrial transcription". The 42 DE genes in "oxidation-reduction process" encompass genes annotated as various cytochromes P450, glutathione peroxidases, dehydrogenases, amine oxidases or peroxidases (file SF1). The 3 DE genes in "mitochondrial cytochrome c oxidase assembly" include two genes annotated as putative RNA binding proteins (the other one is orthologous to the bovine protein "PET100 cytochrome c oxidase chaperone", however it is an uncharacterized protein according to the BLAST with UniRef90 and *I. scapularis* genome; file SF1).

There are also 17 unique enriched MF terms, such as "iron ion binding", "heme binding", "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen" (Table 3.6). The DE genes in those three terms are mostly described as cytochromes P450 (file SF1).

As for the CC enriched terms, there are four unique enriched terms: "extracellular

space", "extracellular region", "mitochondrial membrane" and "myosin complex" (Table 3.6). The terms "extracellular space" and "extracellular region" include DE genes annotated as serpins, serpin precursors or diverse secreted salivary gland peptides (file SF1). "Mitochondrial membrane" DE genes are for instance annotated as ATP synthase E chain, Tim29, putative RNA binding proteins or putative mitochondrial import receptor subunit TOM40 protein 1 (file SF1).

Significantly enriched BP terms solely in D4 (Table 3.6) include "activation of NF-kappaB-inducing kinase activity" and "positive regulation of JNK cascade". For the 6 DE genes which GO annotation are the latter terms (those are the 6 same genes), their BLAST annotation against *I. scapularis* genome is either "Secreted protein" or "Uncharacterized protein", while, in addition, their Uniref90 description indicates they are either "RING-type domain-containing protein" or "Putative TNF receptor-associated factor" (TRAF) (file SF1). Although those terms are not statistically enriched in D0, 7 genes are DE and GO annotated as such, in addition, 3 of them are in fact also DE in D4 (D0_C_N_MT.trinity_TRINITY_DN3254_c1_g2, D4_C_N_OV.trinity_TRINITY_DN23989_ and D4_C_N_OV.trinity_TRINITY_DN26_c0_g1, which are annotated as "RING-type domain-containing protein" for the two former, and uncharacterized for the latter, according to UniRef90 BLAST results; file SF1). It should be noted that two genes corresponding to these BP terms are downregulated in D4 and are annotated as TRAF6-B-like according to Uniref90 (file SF1).

Regarding the MF terms, we find notably "DNA-directed 5'-3' RNA polymerase activity", "acetyltransferase activity" and "zinc ion binding", but with only two DE genes for the two formers (10 for "zinc ion binding") (Table 3.6). The 10 DE genes corresponding to "zinc ion binding" include genes annotated as putative tumor necrosis factor (TNF) receptor-associated factor 4/6 (TRAF4/6) (according to UniRef90 BLAST; file SF1).

As for the CC terms, besides the term "TIM22 mitochondrial import inner membrane insertion complex" which is in common with the D0 time point, we find that the terms "mitochondrial intermembrane space" and "mediator complex" were significantly enriched (Table 3.6). The former comprises two genes annotated as a "mitochondrial import inner membrane translocase subunit Tim8" and an "uncharacterized protein" based on UniRef90 BLAST description (file SF1).

Camera (SF5) and romer (SF4) results notably indicate that the CC term "extracellular space" is enriched for both D0 and D4 time points.

At D4, a specific chitinase, involved in chitin degration (D4_C_N_OV.trinity_TRINITY_DN3451_c0 = IscW_ISCW018618, pathway isc00520) was upregulated. The other annotated chitinases were not found DE.

Downregulated genes (overexpressed in Lab ticks) The next results are again based on topGO analysis (Table 3.6). No common BP terms are enriched between D0 and D4, while "acyl-CoA hydrolase activity" and "TIM22 mitochondrial import inner membrane insertion complex" are the only MF and CC terms found for both time points for the ticks not harbouring *M. mitochondrii*.

At D4, the low number of DE genes still allows the significant enrichment of 6 BP terms (e.g. "vitamin B6 biosynthetic process", "dephosphorylation of RNA polymerase II C-terminal domain" and "mRNA cleavage") but with only 1 DE gene each. The same trend (1 DE gene each time) appears for the 10 other enriched MF terms (e.g. "gamma-glutamylcyclotransferase activity", "coproporphyrinogen oxidase activity", "RNA polymerase II CTD heptapeptide repeat phosphatase activity"). The 2 other enriched CC terms are "host cell nucleus" and "BLOC-1" complex.

At D0, with subsequently more DE genes (Table 3.3), more terms are enriched. The 11 enriched BP terms include "positive regulation of JNK cascade" and "activation of NF-kappaB inducing kinase activity". Among the 9 DE genes for those 2 terms, some are annotated TRAF6, TRAF6-A, TRAF6-B-like or other putative TNF receptor-associated factors (TRAF). None of those 9 DE genes are differentially overexpressed in D4 WT (in the ovary).

10 other MF terms (e.g. "holo-[acyl-carrier-protein] synthase activity", "glutathione transferase activity", "nucleic acid binding" and "methyltransferase activity") and 2 other CC terms ("integral component of membrane" and "ER membrane protein complex") are enriched.

Romer (file SF4) results indicate that very few terms (6 at D4, 14 at D0) are significantly enriched for the Lab ticks, while camera results indicate none (file SF5).

Malpighian tubules

Gene counting In the D4 condition, 784 genes were found to be upregulated for the WT, 636 were downregulated for the WT, while 27,475genes were found to not be DE. In the D0 condition, 3,128 genes were upregulated and 1,678 were downregulated, while 24,089 were found to not be DE (Table 3.3).

Clusterization A heatmap of D0 and D4 DE genes in the Malpighian tubules (Fig. 3.11) allows the visualisation of the expression pattern of all the DE genes within this organ. The two D0_Lab_MT libraries do not cluster unlike what occurs with the ovary (Fig. 3.10A), depicting a singular gene expression pattern that was also observed in the PCA (Fig. S3A).

Gene ontology and gene set analyses

Upregulated genes Enrichment from topGO results (Table 3.6) denotes that 3 BP terms are shared between the two time points ("oxidation-reduction process", "ion transport" and "transmembrane transport") but with around 4 times more DE genes at D0 than at D4. There are no MF nor CC terms shared between D4 and D0.

At D4, 5 other BP terms (e.g. "pathogenesis", "snRNA transport" or "mitochondrial cytochrome c oxidase assembly", all with 2 DE genes each), 9 MF terms (e.g. "heme binding", "iron ion binding", "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen" or "zinc ion binding") and 1 CC term (extracellular region) are enriched (Table 3.6).

Meanwhile, at D0, 18 other BP terms (e.g. "G protein-coupled receptor signaling pathway", "small GTPase mediated signal transduction", "developmental process", "(regulation of) signal transduction" or "cytoskeleton organization"), 17 BP terms (e.g. "guanyl-nucleotide exchange factor activity", "G protein-coupled receptor activity", "hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds", "ATPase-coupled transmembrane transporter activity" or "serine-type peptidase activity") and 3 CC terms ("in-

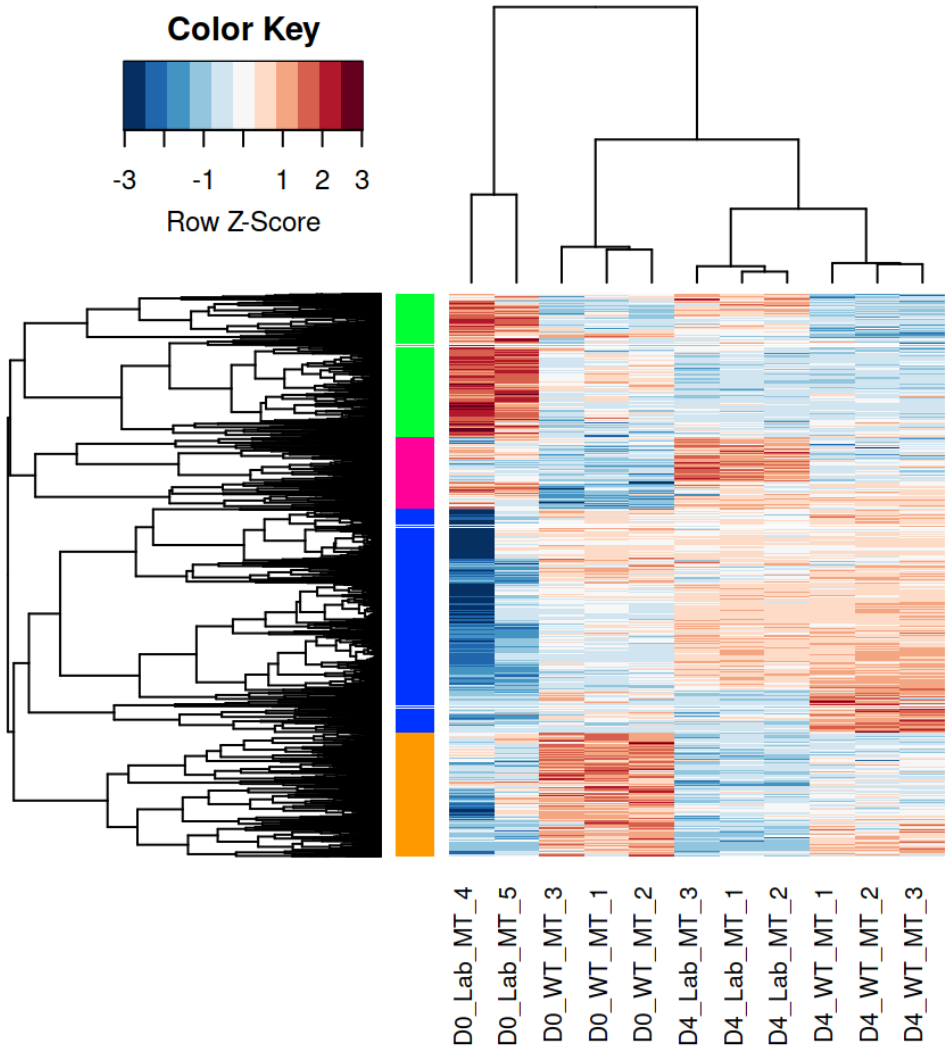

Figure 3.11 – **Heatmap based on the log-count-per-million data of the Malpighian tubules at D0 and D4 for the WT vs Lab comparison**
Low expression values tend towards dark blue while high expression values tend towards dark red. Hierarchical clustering (threshold set at k = 4) of genes is shown by the colours on the left side (blue, orange, pink and green).

tegral component of (plasma) membrane" and "membrane") are enriched (Table 3.6).

Camera test did not find any enriched term whether at D0 or D4 towards the "up" direction (file SF5).

Downregulated genes For the downregulated genes (Table 3.6), topGO results show that no BP nor CC terms are shared at both time points, while 2 MF terms are common between the two strains ("nucleic acid binding" and "coproporphyrinogen oxidase activity").

At D4, 5 BP terms (e.g. "protein processing involved in protein targeting to mitochondrion" and "vitamin B6 biosynthetic process"), 8 MF terms (e.g. "zinc ion binding", "serine-type peptidase activity" or "sulfotransferase activity") and 2 CC terms ("RNA polymerase complex" and "ribosome") are enriched (Table 3.6).

At D0, 26 BP terms (e.g. "activation of NF-kappaB-inducing kinase activity", "positive regulation of JNK cascade", "protein dephosphorylation", "RNA metabolic process" and "DNA recombination"), 14 MF terms (e.g. "acid phosphatase activity", "holo-[acyl-carrier-protein] synthase activity", "transferase activity, transferring phosphorus-containing groups", "methyltransferase activity" or "DNA-binding transcription factor activity") and 5 CC terms (e.g. "DNA packaging complex" and "condensed chromosome") are enriched (Table 3.6).

Camera (file SF5) did not find any enriched term at D0, but found 3 terms at D4, notably "activation of NF-kappaB-inducing kinase activity" and "positive regulation of JNK cascade".

Salivary glands

Gene counting Regarding the salivary glands, the number of unbiased transcripts was 24,304. In the D4 condition, 651 genes were found to be upregulated for the WT, 574 were downregulated for the WT, while 23,079 genes were found to be non significantly differentially expressed. In the D0 condition, 2,003 genes were upregulated and 1,681 were

downregulated, while 20,620 were found to be non significantly differentially expressed (Table 3.3).

Clusterization A heatmap of D0 and D4 DE genes in the salivary glands (Fig. 3.12) allows the visualisation of the expression pattern of all the DE genes within this organ. The three D0_Lab_SG libraries do not cluster and are outgroup, contrary as what happens with the ovary (Fig. 3.10A),

Gene ontology and gene set analyse

Upregulated genes Three BP terms are shared between the D0 and D4 time points, namely "mitigation of host defenses by symbiont", "nucleotide catabolic process" and "proteolysis" (Table 3.6). There are 4 MF terms shared by both time points, to wit "amine binding", "serine-endopeptidase activity", "metalloendopeptidase activity" and "nucleotide binding". No CC terms are common.

The DE genes are the same for the "mitigation of host defenses by symbiont" and "amine binding" terms and comprise genes annotated in majority as "putative salivary lipocalins", "25 kDa salivary gland protein C" and "secreted histamine binding protein". "Nucleotide catabolic process" and "nucleotide binding" genes include 5'-nucleotidases or apyrases . As for "proteolysis" genes, secreted metalloproteases, M13 family peptidases and serine proteases are notably found. The "serine-type endopeptidase activity" genes are mostly constituted by serine proteases and trypsins (file SF1).

At D4, 2 BP terms ("digestion" and "plasma membrane organization"), 8 MF terms (e.g. "serine-type endopeptidase inhibitor activity" and "metallopeptidase activity") and 3 CC terms ("integral component of Golgi membrane", "extracellular space" and "extracellular region") are found only for this time point (Table 3.6).

As for D0, 9 BP terms (e.g. "cytoskeleton organization", "microtubule-based movement" and "regulation of GTPase activity"), 20 MF terms (e.g. "hydrolase activity, acting on ester bonds", "ATP binding", "ATPase activity", "microtubule binding" or "cation binding") and 8 CC terms (e.g. "endoplasmic reticulum" and "integral component of membrane") are exclusive to this time point (Table 3.6).

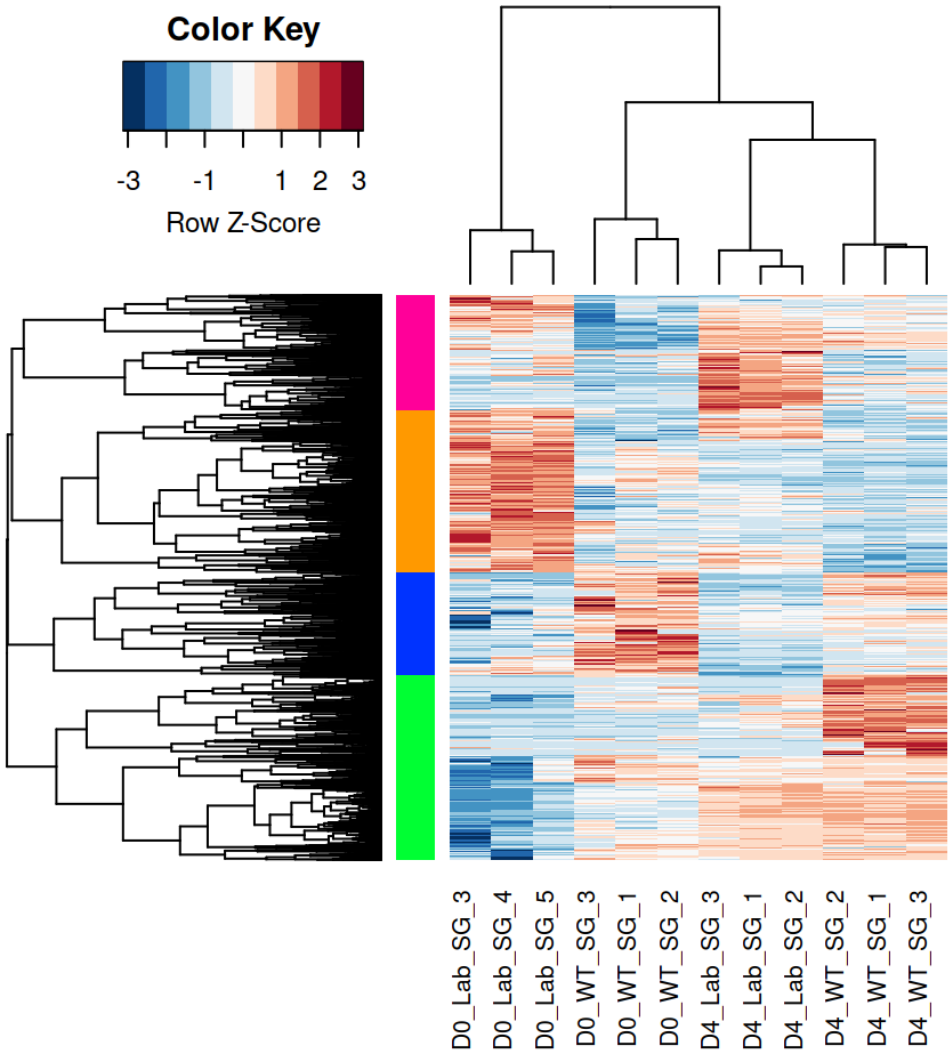

Figure 3.12 – **Heatmap based on the log-count-per-million data of the salivary glands at D0 and D4 for the WT vs Lab comparison**
Low expression values tend towards dark blue while high expression values tend towards dark red. Hierarchical clustering (threshold set at k = 4) of genes is shown by the colours on the left side (blue, orange, pink and green).

Camera results indicate that at D4, the terms "amine binding" and "mitigation of host defenses by symbiont" are enriched in the "up" direction (file SF5) as also found by the topGO results (Table 3.6). At D0, 11 terms are enriched, such as "nucleotide catabolic process", "GTPase activator activity", "ATP binding", "intracellular signal transduction" or "protein phosphorylation" (file SF5).

Downregulated genes Three BP terms ("transmembrane transport", histidine catabolic process", "glutamate metabolic process"), 12 MF terms (e.g. "sulfotransferase activity", "iron ion binding", "chitin binding", "heme binding" or "transmembrane transporter activity" and 1 CC term ("extracellular region") are shared between D0 and D4 time points (Table 3.6).

At D4, 12 BP terms (e.g. "proteolysis", "nucleotide catabolic process", "cellular amide metabolic process", "mitigation of host defenses by symbiont" or "cellular nitrogen compound catabolic process"), 9 MF terms (e.g. "serine-type endopeptidase (inhibitor) activity", "metallo(endo)peptidase activity", "hydrolase activity" or amine binding") and 2 CC terms ("integral component of membrane" and "cytoplasmic dynein complex") are unique to this time point (Table 3.6).

The 14 downregulated DE genes for "mitigation of host defenses by symbiont" are obviously different from the 44 upregulated DE genes (at D4) found above, but also from the 29 DE genes upregulated at D0. Those are annotated as (salivary) lipocalins (UniRef90), zinc finger proteins, secreted salivary gland peptide, 25kDa salivary gland protein C, secreted histamine binding proteins or nymphal histamine binding proteins (according to the *I. scapularis* genome; file SF1).

At D0, 23 BP terms (e.g. "activation of NF-kappaB-inducing kinase activity", "positive regulation of JNK cascade", "peptidoglycan catabolic process", "oxidation-reduction process", "ion transmembrane transport", "translation" or "nitrogen compound metabolic process"), 18 MF terms (e.g. "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen", "glutathione transferase activity", "structural constituent of ribosome", "peptidase activity", "oxidoreductase activity" or "methyltransferase activity") and 5 CC terms (e.g. "dynein complex" and "ribosome") are unique to

this time point (Table 3.6).

The camera results (file SF5) show that the term "transmembrane transporter activity" is enriched in the "down" direction at D4. At D0, 5 terms are enriched, notably "translation", "ribosome", "structural constituent of ribosome" and "sulfotransferase activity".

3.4.6 Outlier discovery

For the WTvsLab comparisons, especially within the ovary and given the relatively small number of DE genes compared to the D4vsD0 comparisons, DE genes were manually examined with their BLAST description. In the genes upregulated in the WT strain, we specifically identified a gene displaying the highest or one of the highest LFC along with a systematic very low p-value, annotated as ricinusin (according to BLASTX against *I. scapularis* genome) in all three tissues and both time points. No GO term is associated, but this particular gene is striking as it is DE in all WTvsLab comparisons (Fig. S5, S6 and S7). We also found a gene annotated as dynactin subunit 3 with the same criterion, likely involved with the cytoskeleton. Additionally, given the strong hypothesis of B vitamins provision by *M. mitochondrii* (most likely biotin and folate), we searched for genes clearly related to B vitamins. None were identified, except a gene that was highly overexpressed in presence of *M. mitochondrii*, annotated as flavin reductase (NADPH) (D4_C_N_OV.trinity_TRINITY_DN1408_c0_g1; file SF1). However, a concrete link with the presence of the symbiont is hard to establish given the *M. mitochondrii* inability to biosynthesize the related B vitamin (riboflavin — B2 vitamin) (Sassera et al., 2011) at the contrary of other tick symbionts (Duron et al., 2018; Gottlieb et al., 2015; Nardi et al., 2021).

3.5 Discussion

We have used a multilayered differential transcriptomics approach to investigate the dynamics of gene expression variation on multiple axes of *I. ricinus* ticks biology, i.e. organs, engorgement level and symbiotic status. First, we characterized the effect of partial engorgement on the expression of genes in three organs, namely the ovary, Malpighian

tubules and salivary glands, which fulfill different functions inside the tick. Second, we seeked to determine the effect of the presence of the main endosymbiont of *I. ricinus*, mainly in the ovary where *M. mitochondrii* principally resides, but also indirectly in the two other organs, where *M. mitochondrii* concentration is lower. The PCAs produced from the mapping of the reads to the *de novo* reference transcriptome (Fig. 3.2, 3.3 and S3) indicate that the organ criterion was the most differential factor, followed by engorgement state. Considering the organs separately, the "symbiont" factor became more evident. As hypothesized, the number of DE genes for engorgement status comparison was much higher overall than the number of DE genes for the symbiont presence/absence comparison.

3.5.1 D4vsD0: comparison of partially engorged ticks versus unfed ticks in all three organs (salivary glands, Malpighian tubules and the ovary)

Interestingly, "protein phosphorylation" (GO:0006468), which encompasses a wide variety of kinases, appears to be the most enriched term in all the tested partially engorged organs regardless the strain, a result already shown, to a lesser extent, in midguts of 3-day fed *I. ricinus* ticks (Perner et al., 2016a), but was not detected by other studies using GO classification and comparing engorgement status (Charrier et al., 2018; Vechtova et al., 2020). Given that these published studies focused solely on fully engorged ticks while ours is on partially fed ticks, one could speculate that the overexpression of various kinases that we detected occurs only during the first, slow phase of feeding (Perner et al., 2016a; Sojka et al., 2013), with the function to anticipate all the morphological changes that occur during the quick and latter phase. It is worth to be noted that Charrier et al., 2018 and Vechtova et al., 2020 studies are on transcriptomes of whole body ticks, while we focused on three specific organs. Even if some biological processes and molecular functions are shared, organ specificities remain.

A novel result of our study is the clear indication that one of the most essential activities of the Malpighian tubules in flat ticks is sulfotransferase activity, as assessed by the highly differentially expressed genes annotated as such and the enrichment tests performed. Sulfotransferases are enzymes responsible for the sulfonation of diverse biomolecules, found in many organism types, from prokaryotes to more complex organisms, likely in-

volved in different biological functions such as drug metabolism, detoxification and hormone regulation (Chapman et al., 2004). Several studies characterized some tick sulfotransferases, from salivary glands (Pichu et al., 2011) or whole body extracts of immature stage (Yalcin et al., 2011), the identified substrates being notably neurotransmitters such as dopamine and octopamine. The BLAST assignments being too imprecise (file SF1), the biological role and the substrates of the detected Malpighian tubules' sulfotransferases remains to be determined.

3.5.2 WTvsLab: comparison of ticks harbouring *M. mitochondrii* endosymbionts versus ticks that do not

Even if *M. mitochondrii* density reaches its maximum level at the end of the tick blood feeding (Sassera et al., 2008), its density is already different by several orders of magnitude at D4 compared to D0. Thus, we chose to analyze such stages in order to avoid the risk of not controlling the latter engorgement phase, as this rapid phase exact timing is impossible to predict and is dependent on each tick. The present section focuses on the results retrieved in the comparison of WT and Lab ovary. In the ovary, the terms 'activation of NF-kappaB-inducing kinase activity' and 'positive regulation of JNK cascade' are upregulated at D4 whereas they are downregulated at D0. These terms could be related to the response induced by a bacterial infection as they are both involved in the innate immune system (Silverman and Maniatis, 2001). In the case of arthropods, while the JNK cascade (part of the IMD pathway) induces a number of complement-like proteins, the activation of NF-kappaB pathway (coined Toll pathway) due to the presence of bacterial compounds (e.g. LPS) in cells induce the production of antimicrobial peptides (AMP), such as drosomycin for Drosophila (Landon et al., 1997), microplusin, ixodesin or ricinusin for ticks (Fogaça et al., 2021; Hajdusek et al., 2013). We could speculate that during the intense *M. mitochondrii* multiplication induced by the blood meal (at D4), these pathways are activated. However, we do not fully understand the cause leading to the activation of such pathways in the unfed ticks not harbouring the symbiont. As mentioned, triggering of these pathways can induce the synthesis of AMP compounds, such as the gene encoding the highly expressed ricinusin in all tissues of WT ticks. The ricinusin peptide sequence is similar to another antimicrobial peptide named microplusin, discovered in Rhipicephalus microplus (Silva et al., 2009). It could be surprising that the control of endosymbiont or pathogen proliferation relies mainly on only one AMP, as

other molecules of the defensin group are known or supposed to have an antimicrobial effect (Nakajima et al., 2002; Nakajima et al., 2003; Wang and Zhu, 2011). However, a recent study indentified a microplusin-like peptide being highly overexpressed in the salivary glands (and midgut) of *Amblyomma aureolatum*, infering a possible tick protection against the rickettsial pathogen *Rickettsia rickettsii*, while the knockdown of the gene had no apparent effect on tick fitness (Martins et al., 2019). Ricinusin and microplusin peptides were notably found to be overexpressed in response upon *Babesia bigemina* infection in *Rhipicephalus annulatus*, but its knockdown did not show any increase or decrease of the pathogen, suggesting a non-mandatory presence of this molecule to control *B. bigemina* infection (Antunes et al., 2012). The striking overexpression of this ricinusin gene could be triggered (in)directly by *M. mitochondrii* at least in the ovary, but why such a phenomenon occurs also in other tissues remains to be determined.

The term 'symbiotic process' is interestingly enriched in the ovary of Lab unfed ticks. This GO term could be related to the presence of any microorganism (virus, bacteria, either commensal, pathogenic or mutualistic). Even if we expect significantly more microorganism diversity and density in the WT ticks and despite the low bacterial mass detected in the Lab ticks (C. Rispe, pers. comm.), we identified an Iflavirus in the Lab strain (Daveu et al., 2021a) which could eventually cause such overexpression of genes associated with this GO term.

In the same way, the term 'negative regulation of apoptotic process' is enriched within the same condition. One could speculate that the absence of *M. mitochondrii* could cause this occurrence, by establishing a link with the symbiont's possible ability to regulate the apoptotic process during mitochondrial invasion (Stavru et al., 2020).

Despite not being enriched solely for this comparison, the MF term "heme binding" was enriched in the unfed WT tick ovary, the DE genes related to this term were mainly annotated as cytochromes P450. This term could refer to *M. mitochondrii* ability to biosynthesize the heme cofactor (Sassera et al., 2011). Heme is a prosthetic group of numerous enzymes involved in a variety of biological processes such as cellular respiration, detoxification of xenobiotics or redox homeostasis (Furuyama et al., 2007; Kořený et al., 2013). Heme has been shown to be acquired during the blood meal via hemoglobin (Perner et al., 2016b) and is believed to be stored in specialized organelles called hemosomes (Lara

et al., 2003; Lara et al., 2005). The vast majority of heme is thus acquired during blood meals, when for instance the female tick ingests more than 100-fold its weight in blood, and transiently used when cellular reactions require. The biosynthesis by *M. mitochondrii* in the ovary is certainly for its own molecular processes, but one could reasonably hypothesize that the symbiont can participate to heme provision to ovarian tick cells at least in a small extent, notably during the off-host life periods (e.g. questing phases), leading to a temporal binding by the mentioned cytochromes. Other ticks symbionts are able to synthesize heme — *Francisella*-like endosymbiont, *Rickettsia* endosymbionts (Driscoll et al., 2017; Gerhart et al., 2016) but not *Coxiella*-like endosymbiont (Moses et al., 2017).

One of the first hypotheses which seemed obvious given the *M. mitochondrii* genome repertoire (Sassera et al., 2011) and due to recent studies on other tick symbionts (Duron et al., 2018; Gottlieb et al., 2015; Kurtti et al., 2015; Nardi et al., 2021; Smith et al., 2015) was the involvement of *M. mitochondrii* in the production of B vitamins. Following this hypothesis, tick genes related to the metabolism of these B vitamins could have exhibited an overexpression in the WT relative to the Lab strain. However, such a hypothesis was finally difficult to be properly tested with our data set in particular due to (i) the lack of precise knowledge concerning tick genes that could be influenced by B vitamins and (ii) the pleiotropic nature of B vitamins as they are involved in numerous metabolic pathways of animal cells (e.g. biotin is typically a coenzyme of carboxylases and folate a methyl donor in nucleic acid and amino acid metabolism; Douglas, 2017). All those possible targets are hardly assignable to distinct GO terms and thus enrichment (even if terms such as “GO:0015878 biotin transport”, “GO:0004075 biotin carboxylase activity”, “ GO:0005542 folic acid binding” or “GO:0071231 cellular response to folic acid” exist), by definition, GO relies on curated annotation and only ca. 40% of our genes were GO assigned. Nonetheless, even in the absence of *M. mitochondrii*, as the Lab strain is still able to survive and without any real certainty of the microbiome composition of this Lab strain, B vitamins may be provided by potential alternative sources other than *M. mitochondrii* (Díaz-Sánchez et al., 2019; Guizzo et al., 2020; Lejal et al., 2020a; Rio et al., 2016). However, such an explanation would highly hamper the identification of tick genes affected by the *M. mitochondrii* absence/presence.

Abbreviations

BUSCO: Benchmarking Universal Single-Copy Orthologs BP: Biological Process CC: Cellular Component CPM: Count Per Million DE: differentially expressed/differential expression GO: Gene Ontology KEGG: Kyoto Encyclopedia of Genes and Genomes LFC: log2 fold-change MDS: multi-dimentional scalling MF: Molecular Function MT: Malpighian tubules NF: nuclear factor ORF: Open Reading Frame OV: ovary SG: salivary glands TNF: tumor necrosis factor TRAF: TNF receptor-associated factor WT: Wild type

Acknowledgement

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3.6 Supporting information

Tables 3.4—3.5—3.6—3.7 presented in this manuscript can be viewed in their most updated version at (along with other analyses not presented) at:
`https://drive.google.com/drive/folders/1JSjs_TKMPvA2ZOJjxsOja4ziM5BZftf4?usp=sharing`
or downloaded as a zip file (created on 19th May 2021) at:
`https://drive.google.com/file/d/1Hj2aNKCfGGusaXSsMMV-8P7Y2jBiLdfy/view?usp=sharing`

Individual tables can be viewed and downloaded using the different links below (see the different captions).

Table 3.4 – **Ten (max) most significantly enriched GO terms (lowest p-values) using topGO for each strain (WT, Lab) or regardless of the strain (global) for the D4 vs D0 comparison in each tissue (OV, MT, SG) for each ontology (BP, MF, CC)**
This table can be downloaded at: `https://drive.google.com/file/d/1G_lOAGxh9qJ6yipgZTAd2NvKaP5qImqR/view?usp=sharing`

GO.ID	Term	Annotated	Significant	Expected	p-value
OV.UpWT.D4vsD0.BP					
GO:0006468	protein phosphorylation	459	146	88.2	1.5E-18
GO:0007165	signal transduction	678	212	130.29	9E-11
GO:0006355	regulation of transcription, DNA-templated	495	146	95.12	2.2E-09
GO:0035556	intracellular signal transduction	387	108	74.37	3.3E-07
GO:0007010	cytoskeleton organization	147	59	28.25	5.2E-07
GO:0015074	DNA integration	55	25	10.57	7.9E-06
GO:0009765	photosynthesis, light harvesting	13	10	2.5	1.1E-05
GO:0007155	cell adhesion	47	25	9.03	4.6E-05
GO:0050808	synapse organization	8	7	1.54	6.4E-05
GO:0006397	mRNA processing	167	38	32.09	0.00014
OV.UpWT.D4vsD0.MF					
GO:0005515	protein binding	2743	755	551.26	2.1E-19
GO:0004674	protein serine/threonine kinase activity	100	50	20.1	4E-10
GO:0005524	ATP binding	802	229	161.18	1.4E-09
GO:0004190	aspartic-type endopeptidase activity	28	18	5.63	4.4E-07

GO:0005509	calcium ion binding	192	66	38.59	2.2E-06
GO:0051015	actin filament binding	33	19	6.63	2.4E-06
GO:0008017	microtubule binding	51	25	10.25	3.4E-06
GO:0005085	guanyl-nucleotide exchange factor activity	71	35	14.27	8.2E-06
GO:0004672	protein kinase activity	314	129	63.1	1.1E-05
GO:0005319	lipid transporter activity	36	18	7.23	1.3E-05
OV.UpWT.D4vsD0.CC					
GO:0005911	cell-cell junction	6	6	1.27	8.7E-05
GO:0016459	myosin complex	21	12	4.43	0.00031
GO:0005856	cytoskeleton	207	57	43.65	0.00057
GO:0005886	plasma membrane	99	38	20.88	0.00354
GO:0005667	transcription regulator complex	76	18	16.03	0.0038
GO:0016020	membrane	1458	315	307.44	0.004
GO:0005615	extracellular space	57	21	12.02	0.00434
GO:0005819	spindle	15	7	3.16	0.0059
GO:0030015	CCR4-NOT core complex	7	5	1.48	0.00591
GO:0070161	anchoring junction	9	9	1.9	0.00918
OV.UpLab.D4vsD0.BP					
GO:0006468	protein phosphorylation	459	152	94.57	7.1E-18
GO:0006355	regulation of transcription, DNA-templated	495	151	101.99	2.5E-10
GO:0007165	signal transduction	678	226	139.69	3.6E-10
GO:0035556	intracellular signal transduction	387	118	79.74	1.1E-08
GO:0007155	cell adhesion	47	29	9.68	2.5E-07

GO:0007010	cytoskeleton organization	147	64	30.29	1E-06
GO:0015074	DNA integration	55	26	11.33	8.1E-06
GO:0030036	actin cytoskeleton organization	82	28	16.9	3.6E-05
GO:0050808	synapse organization	8	7	1.65	0.0001
GO:0051056	regulation of small GTPase mediated signal transduction	28	10	5.77	0.0001
OV.UpLab.D4vsD0.MF					
GO:0005515	protein binding	2743	844	606.57	1.2E-24
GO:0005524	ATP binding	802	271	177.35	1.6E-15
GO:0005509	calcium ion binding	192	81	42.46	2.7E-10
GO:0008017	microtubule binding	51	32	11.28	4.8E-10
GO:0005085	guanyl-nucleotide exchange factor activity	71	41	15.7	8.5E-10
GO:0004674	protein serine/threonine kinase activity	100	46	22.11	1.1E-07
GO:0004672	protein kinase activity	314	134	69.44	3.4E-07
GO:0003774	motor activity	43	27	9.51	1.9E-06
GO:0051015	actin filament binding	33	20	7.3	2E-06
GO:0003779	actin binding	102	51	22.56	8.6E-06
OV.UpLab.D4vsD0.CC					
GO:0016459	myosin complex	21	15	4.47	1.1E-06
GO:0005856	cytoskeleton	207	71	44.03	0.00014
GO:0005634	nucleus	765	176	162.74	0.00016
GO:0005886	plasma membrane	99	38	21.06	0.00129
GO:0045202	synapse	8	6	1.7	0.00172

GO:0005911	cell-cell junction	6	5	1.28	0.00213
GO:0030015	CCR4-NOT core complex	7	5	1.49	0.00615
GO:0070161	anchoring junction	9	8	1.91	0.00946
GO:0030127	COPII vesicle coat	8	5	1.7	0.01355
GO:0005667	transcription regulator complex	76	18	16.17	0.01831
OV.UpGlobal.D4vsD0.BP					
GO:0006468	protein phosphorylation	459	149	96.19	7.2E-16
GO:0006355	regulation of transcription, DNA-templated	495	152	103.74	6.1E-09
GO:0007165	signal transduction	678	219	142.09	2.7E-08
GO:0035556	intracellular signal transduction	387	112	81.11	2E-07
GO:0007155	cell adhesion	47	29	9.85	3.4E-07
GO:0015074	DNA integration	55	28	11.53	7.9E-07
GO:0007010	cytoskeleton organization	147	64	30.81	1.2E-06
GO:0006869	lipid transport	50	22	10.48	4.2E-06
GO:0009765	photosynthesis, light harvesting	13	10	2.72	2.4E-05
GO:0007411	axon guidance	18	12	3.77	3.6E-05
OV.UpGlobal.D4vsD0.MF					
GO:0005515	protein binding	2743	818	602.58	1.6E-21
GO:0005524	ATP binding	802	261	176.18	3.6E-13
GO:0008017	microtubule binding	51	30	11.2	1.3E-08
GO:0004674	protein serine/threonine kinase activity	100	46	21.97	2.5E-08
GO:0005509	calcium ion binding	192	73	42.18	2.6E-07
GO:0051015	actin filament binding	33	21	7.25	3E-07

GO:0004672	protein kinase activity	314	131	68.98	1.4E-06
GO:0005085	guanyl-nucleotide exchange factor activity	71	36	15.6	2.6E-06
GO:0003779	actin binding	102	52	22.41	7.4E-06
GO:0004190	aspartic-type endopeptidase activity	28	17	6.15	1.1E-05
OV.UpGlobal.D4vsD0.CC					
GO:0016459	myosin complex	21	14	4.76	2E-05
GO:0005911	cell-cell junction	6	6	1.36	0.00013
GO:0005856	cytoskeleton	207	67	46.9	0.00119
GO:0005615	extracellular space	57	23	12.91	0.00194
GO:0005886	plasma membrane	99	38	22.43	0.00266
GO:0005667	transcription regulator complex	76	19	17.22	0.00702
GO:0005819	spindle	15	8	3.4	0.00815
GO:0030015	CCR4-NOT core complex	7	5	1.59	0.00821
GO:0070161	anchoring junction	9	9	2.04	0.01141
GO:0045202	synapse	8	5	1.81	0.01782
OV.DownWT.D4vsD0.BP					
GO:0016226	iron-sulfur cluster assembly	17	10	2.05	5.3E-06
GO:0032481	positive regulation of type I interferon production	6	5	0.72	0.00014
GO:0002218	activation of innate immune response	6	5	0.72	0.00014
GO:0006744	ubiquinone biosynthetic process	13	7	1.56	0.00032
GO:0036265	RNA (guanine-N7)-methylation	5	4	0.6	0.00094

GO:0010038	response to metal ion	13	6	1.56	0.00102
GO:0006627	protein processing involved in protein targeting to mitochondrion	8	5	0.96	0.00102
GO:0006390	mitochondrial transcription	8	5	0.96	0.00102
GO:0051186	cofactor metabolic process	132	32	15.89	0.00166
GO:0033013	tetrapyrrole metabolic process	16	6	1.93	0.00173
OV.DownWT.D4vsD0.MF					
GO:0004298	threonine-type endopeptidase activity	24	11	2.79	3E-05
GO:0004726	non-membrane spanning protein tyrosine phosphatase activity	11	7	1.28	6.2E-05
GO:0004526	ribonuclease P activity	6	5	0.7	0.00012
GO:0005506	iron ion binding	190	40	22.12	0.00032
GO:0004844	uracil DNA N-glycosylase activity	7	5	0.82	0.00036
GO:0003676	nucleic acid binding	1751	262	203.87	0.00057
GO:0003993	acid phosphatase activity	15	7	1.75	0.00079
GO:0051087	chaperone binding	8	5	0.93	0.00088
GO:0016860	intramolecular oxidoreductase activity	21	6	2.45	0.00157
GO:0016274	protein-arginine N-methyltransferase activity	13	6	1.51	0.00204
OV.DownWT.D4vsD0.CC					
GO:0008180	COP9 signalosome	14	8	1.77	9.1E-05
GO:0005681	spliceosomal complex	29	11	3.66	0.00047
GO:0005730	nucleolus	30	11	3.79	0.00066

GO:0030915	Smc5-Smc6 complex	11	6	1.39	0.00103
GO:0005739	mitochondrion	260	59	32.81	0.00105
GO:0005743	mitochondrial inner membrane	94	25	11.86	0.0011
GO:0019773	proteasome core complex, alpha-subunit complex	10	5	1.26	0.00458
GO:0042721	TIM22 mitochondrial import inner membrane insertion complex	8	4	1.01	0.01154
GO:0005839	proteasome core complex	23	10	2.9	0.0164
GO:0000178	exosome (RNase complex)	13	5	1.64	0.01695
OV.DownLab.D4vsD0.BP					
GO:0016226	iron-sulfur cluster assembly	17	14	2.07	6.9E-11
GO:0055070	copper ion homeostasis	8	7	0.97	2.8E-06
GO:0046081	dUTP catabolic process	6	6	0.73	3.2E-06
GO:0006226	dUMP biosynthetic process	6	6	0.73	3.2E-06
GO:0032481	positive regulation of type I interferon production	6	5	0.73	0.00014
GO:0002218	activation of innate immune response	6	5	0.73	0.00014
GO:0000387	spliceosomal snRNP assembly	23	10	2.8	0.00018
GO:0006352	DNA-templated transcription, initiation	52	17	6.34	0.00041
GO:0016070	RNA metabolic process	1144	190	139.4	0.00077
GO:0032981	mitochondrial respiratory chain complex I assembly	11	6	1.34	0.00086
OV.DownLab.D4vsD0.MF					

GO:0004298	threonine-type endopeptidase activity	24	13	2.69	3.1E-07
GO:0046982	protein heterodimerization activity	71	23	7.95	1.4E-06
GO:0004170	dUTP diphosphatase activity	6	6	0.67	1.9E-06
GO:0004526	ribonuclease P activity	6	5	0.67	9.5E-05
GO:0051536	iron-sulfur cluster binding	57	22	6.38	0.00035
GO:0004726	non-membrane spanning protein tyrosine phosphatase activity	11	6	1.23	0.00055
GO:0003824	catalytic activity	4621	548	517.43	0.00061
GO:0003993	acid phosphatase activity	15	7	1.68	0.00062
GO:0004109	coproporphyrinogen oxidase activity	5	4	0.56	0.00071
GO:0000179	rRNA (adenine-N6,N6-)-dimethyltransferase activity	8	5	0.9	0.00073
OV.DownLab.D4vsD0.CC					
GO:0030880	RNA polymerase complex	49	10	6.69	0.00034
GO:0005839	proteasome core complex	23	12	3.14	0.00066
GO:0005743	mitochondrial inner membrane	94	29	12.84	0.00203
GO:0016592	mediator complex	41	13	5.6	0.00225
GO:0005739	mitochondrion	260	69	35.51	0.00234
GO:0005885	Arp2/3 protein complex	12	6	1.64	0.00281
GO:0019773	proteasome core complex, alpha-subunit complex	10	5	1.37	0.0065
GO:0008180	COP9 signalosome	14	6	1.91	0.00716
GO:0005681	spliceosomal complex	29	9	3.96	0.01222

GO:0032993	protein-DNA complex	35	11	4.78	0.02003
OV.DownGlobal.D4vsD0.BP					
GO:0016226	iron-sulfur cluster assembly	17	12	2.05	3.1E-08
GO:0055070	copper ion homeostasis	8	6	0.97	6.8E-05
GO:0032481	positive regulation of type I interferon production	6	5	0.72	0.00014
GO:0046081	dUTP catabolic process	6	5	0.72	0.00014
GO:0006226	dUMP biosynthetic process	6	5	0.72	0.00014
GO:0002218	activation of innate immune response	6	5	0.72	0.00014
GO:0006284	base-excision repair	17	8	2.05	0.00039
GO:0032981	mitochondrial respiratory chain complex I assembly	11	6	1.33	0.00082
GO:0010038	response to metal ion	13	6	1.57	0.00103
GO:0006627	protein processing involved in protein targeting to mitochondrion	8	5	0.97	0.00103
OV.DownGlobal.D4vsD0.MF					
GO:0004298	threonine-type endopeptidase activity	24	12	2.74	3.3E-06
GO:0004726	non-membrane spanning protein tyrosine phosphatase activity	11	7	1.25	5.4E-05
GO:0004170	dUTP diphosphatase activity	6	5	0.68	0.0001
GO:0004526	ribonuclease P activity	6	5	0.68	0.0001
GO:0046982	protein heterodimerization activity	71	19	8.09	0.00027
GO:0004844	uracil DNA N-glycosylase activity	7	5	0.8	0.00033

GO:0003725	double-stranded RNA binding	7	5	0.8	0.00033
GO:0003993	acid phosphatase activity	15	7	1.71	0.00069
GO:0008897	holo-[acyl-carrier-protein] synthase activity	5	4	0.57	0.00076
GO:0003747	translation release factor activity	8	5	0.91	0.0008
OV.DownGlobal.D4vsD0.CC					
GO:0005743	mitochondrial inner membrane	94	29	12.34	0.00036
GO:0005730	nucleolus	30	10	3.94	0.00357
GO:0005839	proteasome core complex	23	11	3.02	0.00362
GO:0019773	proteasome core complex, alpha-subunit complex	10	5	1.31	0.00546
GO:0008180	COP9 signalosome	14	6	1.84	0.00587
GO:0030880	RNA polymerase complex	49	11	6.43	0.00802
GO:0030915	Smc5-Smc6 complex	11	5	1.44	0.00894
GO:0042721	TIM22 mitochondrial import inner membrane insertion complex	8	4	1.05	0.01328
GO:0016592	mediator complex	41	11	5.38	0.0139
GO:0005739	mitochondrion	260	61	34.13	0.01861
MT.UpWT.D4vsD0.BP					
GO:0006468	protein phosphorylation	459	121	85.58	7.8E-12
GO:0006412	translation	478	116	89.13	3.4E-06
GO:0007165	signal transduction	678	181	126.42	4.2E-06
GO:0007264	small GTPase mediated signal transduction	84	34	15.66	2E-05
GO:0035556	intracellular signal transduction	387	93	72.16	2E-05

GO:0034968	histone lysine methylation	18	11	3.36	8E-05
GO:0099504	synaptic vesicle cycle	5	5	0.93	0.00022
GO:0007017	microtubule-based process	149	57	27.78	0.00024
GO:0006355	regulation of transcription, DNA-templated	495	118	92.29	0.0003
GO:0006471	protein ADP-ribosylation	6	5	1.12	0.00114
MT.UpWT.D4vsD0.MF					
GO:0005524	ATP binding	802	237	152.86	5E-14
GO:0008017	microtubule binding	51	30	9.72	3.6E-10
GO:0005515	protein binding	2743	663	522.81	8.2E-09
GO:0005200	structural constituent of cytoskeleton	42	24	8.01	4.6E-08
GO:0004674	protein serine/threonine kinase activity	100	42	19.06	4.5E-06
GO:0003735	structural constituent of ribosome	359	103	68.42	4.5E-06
GO:0018024	histone-lysine N-methyltransferase activity	20	13	3.81	8.5E-06
GO:0017048	Rho GTPase binding	20	11	3.81	2.9E-05
GO:0003777	microtubule motor activity	22	13	4.19	3.7E-05
GO:0005319	lipid transporter activity	36	20	6.86	7.1E-05
MT.UpWT.D4vsD0.CC					
GO:0005840	ribosome	357	103	71.67	8.5E-08
GO:0005874	microtubule	56	29	11.24	1E-07
GO:0005667	transcription regulator complex	76	21	15.26	0.00014
GO:0005819	spindle	15	7	3.01	0.0047

GO:0044815	DNA packaging complex	33	12	6.62	0.00797
GO:0070161	anchoring junction	9	7	1.81	0.00797
GO:0005643	nuclear pore	30	12	6.02	0.00951
GO:0030127	COPII vesicle coat	8	5	1.61	0.0105
GO:0016459	myosin complex	21	9	4.22	0.01451
GO:0000159	protein phosphatase type 2A complex	6	4	1.2	0.01711
MT.UpLab.D4vsD0.BP					
GO:0006412	translation	478	163	113.63	3.3E-13
GO:0006468	protein phosphorylation	459	133	109.12	1.3E-10
GO:0007165	signal transduction	678	215	161.18	5.9E-08
GO:0006355	regulation of transcription, DNA-templated	495	152	117.67	1.4E-05
GO:0007155	cell adhesion	47	26	11.17	6.4E-05
GO:0006546	glycine catabolic process	6	6	1.43	0.00018
GO:0035556	intracellular signal transduction	387	99	92	0.00031
GO:0007018	microtubule-based movement	36	15	8.56	0.00074
GO:0007010	cytoskeleton organization	147	61	34.95	0.00075
GO:0044087	regulation of cellular component biogenesis	30	10	7.13	0.00076
MT.UpLab.D4vsD0.MF					
GO:0003735	structural constituent of ribosome	359	148	87.71	7.3E-13
GO:0005524	ATP binding	802	279	195.93	4.5E-12
GO:0008017	microtubule binding	51	32	12.46	6.8E-09
GO:0005515	protein binding	2743	798	670.13	5.8E-06

GO:0051015	actin filament binding	33	20	8.06	1E-05
GO:0004674	protein serine/threonine kinase activity	100	43	24.43	1.5E-05
GO:0004672	protein kinase activity	314	129	76.71	3.4E-05
GO:0003774	motor activity	43	27	10.51	4.9E-05
GO:0005085	guanyl-nucleotide exchange factor activity	71	32	17.35	0.00022
GO:0004713	protein tyrosine kinase activity	33	18	8.06	0.00029
MT.UpLab.D4vsD0.CC					
GO:0005840	ribosome	357	146	93.43	1.3E-10
GO:0016459	myosin complex	21	14	5.5	0.00011
GO:0005667	transcription regulator complex	76	21	19.89	0.00807
GO:0005643	nuclear pore	30	14	7.85	0.01213
GO:0030015	CCR4-NOT core complex	7	5	1.83	0.01571
GO:0005819	spindle	15	7	3.93	0.01572
GO:0042025	host cell nucleus	139	48	36.38	0.01615
GO:0000785	chromatin	80	30	20.94	0.01751
GO:0070161	anchoring junction	9	6	2.36	0.01781
GO:0031012	extracellular matrix	5	4	1.31	0.01848
MT.UpGlobal.D4vsD0.BP					
GO:0006468	protein phosphorylation	459	132	101.81	1.1E-11
GO:0006412	translation	478	145	106.03	4.5E-10
GO:0007165	signal transduction	678	210	150.39	4.8E-06
GO:0035556	intracellular signal transduction	387	102	85.84	9.2E-06
GO:0007155	cell adhesion	47	24	10.43	9E-05

GO:0006355	regulation of transcription, DNA-templated	495	142	109.8	9.3E-05
GO:0007264	small GTPase mediated signal transduction	84	35	18.63	0.0001
GO:0034968	histone lysine methylation	18	11	3.99	0.00041
GO:0044087	regulation of cellular component biogenesis	30	11	6.65	0.00053
GO:0099504	synaptic vesicle cycle	5	5	1.11	0.00053
MT.UpGlobal.D4vsD0.MF					
GO:0005524	ATP binding	802	265	181.47	1.2E-12
GO:0005515	protein binding	2743	771	620.67	2E-09
GO:0003735	structural constituent of ribosome	359	129	81.23	4E-09
GO:0008017	microtubule binding	51	31	11.54	5E-09
GO:0004674	protein serine/threonine kinase activity	100	47	22.63	1.5E-08
GO:0005085	guanyl-nucleotide exchange factor activity	71	34	16.07	1.8E-05
GO:0003777	microtubule motor activity	22	14	4.98	4.4E-05
GO:0018024	histone-lysine N-methyltransferase activity	20	13	4.53	6E-05
GO:0004672	protein kinase activity	314	128	71.05	7E-05
GO:0005200	structural constituent of cytoskeleton	42	21	9.5	9.1E-05
MT.UpGlobal.D4vsD0.CC					
GO:0005840	ribosome	357	128	85.83	9.7E-08

GO:0005874	microtubule	56	27	13.46	6.3E-05
GO:0016459	myosin complex	21	12	5.05	0.0011
GO:0005667	transcription regulator complex	76	21	18.27	0.0037
GO:0016514	SWI/SNF complex	6	5	1.44	0.0038
GO:0005643	nuclear pore	30	14	7.21	0.0055
GO:0005819	spindle	15	7	3.61	0.0107
GO:0030015	CCR4-NOT core complex	7	5	1.68	0.0107
GO:0070161	anchoring junction	9	7	2.16	0.0137
GO:0070013	intracellular organelle lumen	309	56	74.29	0.0149
MT.DownWT.D4vsD0.BP					
GO:0055114	oxidation-reduction process	650	145	80.89	9.4E-17
GO:0055085	transmembrane transport	582	93	72.42	0.001
GO:0035721	intraciliary retrograde transport	5	4	0.62	0.0011
GO:0035735	intraciliary transport involved in cilium assembly	5	4	0.62	0.0011
GO:0006390	mitochondrial transcription	8	5	1	0.0012
GO:0006826	iron ion transport	8	5	1	0.0012
GO:0001558	regulation of cell growth	17	7	2.12	0.0028
GO:0055082	cellular chemical homeostasis	26	8	3.24	0.0029
GO:0106035	protein maturation by [4Fe-4S] cluster transfer	6	4	0.75	0.0029
GO:0006526	arginine biosynthetic process	6	4	0.75	0.0029
MT.DownWT.D4vsD0.MF					
GO:0005506	iron ion binding	190	86	23.71	9.3E-29

GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	194	78	24.21	3.1E-25
GO:0020037	heme binding	163	69	20.34	7.1E-22
GO:0008146	sulfotransferase activity	115	40	14.35	2.9E-10
GO:0004497	monooxygenase activity	37	13	4.62	3.9E-05
GO:0022857	transmembrane transporter activity	614	94	76.61	0.00013
GO:0008083	growth factor activity	23	9	2.87	0.00113
GO:0004869	cysteine-type endopeptidase inhibitor activity	19	8	2.37	0.00122
GO:0005520	insulin-like growth factor binding	16	7	2	0.0019
GO:0004104	cholinesterase activity	18	7	2.25	0.00422
MT.DownWT.D4vsD0.CC					
GO:0005576	extracellular region	189	42	23.15	0.00016
GO:0030173	integral component of Golgi membrane	6	4	0.73	0.00272
GO:0016021	integral component of membrane	712	109	87.2	0.00391
GO:0005868	cytoplasmic dynein complex	10	5	1.22	0.00401
GO:0005730	nucleolus	30	9	3.67	0.00767
GO:0032040	small-subunit processome	9	4	1.1	0.01688
GO:0031966	mitochondrial membrane	127	14	15.55	0.01747
GO:0005681	spliceosomal complex	29	8	3.55	0.01975
GO:0008180	COP9 signalosome	14	5	1.71	0.02101
GO:0000124	SAGA complex	15	5	1.84	0.02842
MT.DownLab.D4vsD0.BP					

GO:0055114	oxidation-reduction process	650	147	102.99	4.1E-09
GO:0046330	positive regulation of JNK cascade	140	50	22.18	5.3E-09
GO:0007250	activation of NF-kappaB-inducing kinase activity	140	50	22.18	5.3E-09
GO:0016226	iron-sulfur cluster assembly	17	10	2.69	6.4E-05
GO:0035721	intraciliary retrograde transport	5	5	0.79	9.9E-05
GO:0035735	intraciliary transport involved in cilium assembly	5	5	0.79	9.9E-05
GO:0006801	superoxide metabolic process	34	14	5.39	0.00035
GO:0018216	peptidyl-arginine methylation	14	8	2.22	0.00047
GO:0016070	RNA metabolic process	1144	216	181.25	0.00241
GO:0060271	cilium assembly	16	11	2.54	0.00345
MT.DownLab.D4vsD0.MF					
GO:0005506	iron ion binding	190	87	29.99	4.4E-21
GO:0020037	heme binding	163	72	25.73	4.3E-18
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	194	77	30.62	1.1E-16
GO:0005126	cytokine receptor binding	6	6	0.95	1.5E-05
GO:0008168	methyltransferase activity	214	62	33.78	4.8E-05
GO:0008146	sulfotransferase activity	115	35	18.15	0.0001
GO:0003993	acid phosphatase activity	15	9	2.37	0.00012
GO:0016274	protein-arginine N-methyltransferase activity	13	8	2.05	0.00023

GO:0004726	non-membrane spanning protein tyrosine phosphatase activity	11	7	1.74	0.00044
GO:0004526	ribonuclease P activity	6	5	0.95	0.00051
MT.DownLab.D4vsD0.CC					
GO:0008180	COP9 signalosome	14	8	2.16	0.00039
GO:0005730	nucleolus	30	11	4.63	0.00357
GO:0016591	RNA polymerase II, holoenzyme	37	12	5.71	0.00842
GO:0005681	spliceosomal complex	29	10	4.48	0.00885
GO:0000808	origin recognition complex	10	5	1.54	0.01108
GO:0005868	cytoplasmic dynein complex	10	5	1.54	0.01108
GO:0090575	RNA polymerase II transcription regulator complex	42	12	6.49	0.01292
GO:0000813	ESCRT I complex	8	4	1.24	0.02347
GO:0031011	Ino80 complex	16	6	2.47	0.02659
GO:0032993	protein-DNA complex	35	11	5.4	0.02827
MT.DownGlobal.D4vsD0.BP					
GO:0055114	oxidation-reduction process	650	158	94.5	2.9E-15
GO:0046330	positive regulation of JNK cascade	140	39	20.35	2.8E-05
GO:0007250	activation of NF-kappaB-inducing kinase activity	140	39	20.35	2.8E-05
GO:0035721	intraciliary retrograde transport	5	5	0.73	6.4E-05
GO:0035735	intraciliary transport involved in cilium assembly	5	5	0.73	6.4E-05
GO:0006801	superoxide metabolic process	34	14	4.94	0.00014
GO:0001558	regulation of cell growth	17	8	2.47	0.00139

GO:0006826	iron ion transport	8	5	1.16	0.00246
GO:0055082	cellular chemical homeostasis	26	8	3.78	0.0052
GO:0106035	protein maturation by [4Fe-4S] cluster transfer	6	4	0.87	0.00522
MT.DownGlobal.D4vsD0.MF					
GO:0005506	iron ion binding	190	96	28.62	8.4E-30
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	194	87	29.23	4.2E-27
GO:0020037	heme binding	163	78	24.56	1.9E-23
GO:0008146	sulfotransferase activity	115	48	17.33	2.6E-11
GO:0005126	cytokine receptor binding	6	6	0.9	1.2E-05
GO:0003993	acid phosphatase activity	15	9	2.26	8.3E-05
GO:0004497	monooxygenase activity	37	13	5.57	0.00025
GO:0004726	non-membrane spanning protein tyrosine phosphatase activity	11	7	1.66	0.00033
GO:0004104	cholinesterase activity	18	9	2.71	0.00052
GO:0004869	cysteine-type endopeptidase inhibitor activity	19	9	2.86	0.00086
MT.DownGlobal.D4vsD0.CC					
GO:0005576	extracellular region	189	49	25.86	0.00021
GO:0005868	cytoplasmic dynein complex	10	6	1.37	0.00082
GO:0090575	RNA polymerase II transcription regulator complex	42	13	5.75	0.00121
GO:0030914	STAGA complex	5	4	0.68	0.00155

GO:0005615	extracellular space	57	16	7.8	0.00308
GO:0005681	spliceosomal complex	29	10	3.97	0.00368
GO:0005730	nucleolus	30	10	4.11	0.00486
GO:0016591	RNA polymerase II, holoenzyme	37	11	5.06	0.00966
GO:0032040	small-subunit processome	9	4	1.23	0.02475
GO:0000808	origin recognition complex	10	4	1.37	0.03688
SG.UpWT.D4vsD0.BP					
GO:0030682	mitigation of host defenses by symbiont	173	82	35.69	1.5E-15
GO:0006468	protein phosphorylation	459	115	94.7	5.4E-10
GO:0006508	proteolysis	707	193	145.86	7.1E-08
GO:0035556	intracellular signal transduction	387	91	79.84	4.9E-07
GO:0007165	signal transduction	678	192	139.88	2.9E-06
GO:0007155	cell adhesion	47	25	9.7	3.1E-05
GO:0007010	cytoskeleton organization	147	45	30.33	0.00028
GO:0030036	actin cytoskeleton organization	82	26	16.92	0.0012
GO:0043547	positive regulation of GTPase activity	13	8	2.68	0.00153
GO:0006013	mannose metabolic process	6	5	1.24	0.00185
SG.UpWT.D4vsD0.MF					
GO:0043176	amine binding	173	82	36.83	1.3E-14
GO:0042302	structural constituent of cuticle	31	23	6.6	4.2E-10
GO:0004674	protein serine/threonine kinase activity	100	48	21.29	1.4E-07

GO:0004867	serine-type endopeptidase inhibitor activity	153	60	32.57	2.9E-07
GO:0008237	metallopeptidase activity	304	107	64.72	1.8E-05
GO:0045296	cadherin binding	7	7	1.49	2E-05
GO:0004222	metalloendopeptidase activity	219	73	46.62	2E-05
GO:0000166	nucleotide binding	1286	302	273.78	2.1E-05
GO:0004713	protein tyrosine kinase activity	33	18	7.03	5.5E-05
GO:0017048	Rho GTPase binding	20	12	4.26	8E-05
SG.UpWT.D4vsD0.CC					
GO:0005840	ribosome	357	94	71.67	0.00011
GO:0005886	plasma membrane	99	40	19.87	0.00018
GO:0016459	myosin complex	21	11	4.22	0.00097
GO:0005856	cytoskeleton	207	46	41.55	0.00128
GO:0005911	cell-cell junction	6	5	1.2	0.00161
GO:0043229	intracellular organelle	1855	341	372.38	0.00227
GO:0005615	extracellular space	57	20	11.44	0.00558
GO:0031012	extracellular matrix	5	4	1	0.00678
GO:0070161	anchoring junction	9	8	1.81	0.00794
GO:0070013	intracellular organelle lumen	309	41	62.03	0.00891
SG.UpLab.D4vsD0.BP					
GO:0006468	protein phosphorylation	459	137	123.54	9.4E-08
GO:0007165	signal transduction	678	247	182.48	1.6E-07
GO:0007155	cell adhesion	47	31	12.65	1.4E-06
GO:0006508	proteolysis	707	234	190.28	5.5E-06
GO:0035556	intracellular signal transduction	387	112	104.16	6.4E-06

GO:0007264	small GTPase mediated signal transduction	84	41	22.61	2.5E-05
GO:0009166	nucleotide catabolic process	63	31	16.96	0.00012
GO:0007010	cytoskeleton organization	147	57	39.56	0.00022
GO:0043547	positive regulation of GTPase activity	13	10	3.5	0.00024
GO:0006471	protein ADP-ribosylation	6	6	1.61	0.00038
SG.UpLab.D4vsD0.MF					
GO:0005524	ATP binding	802	297	222.99	2E-09
GO:0004222	metalloendopeptidase activity	219	99	60.89	2.1E-08
GO:0005515	protein binding	2743	911	762.67	7.2E-08
GO:0008017	microtubule binding	51	32	14.18	1.9E-07
GO:0000166	nucleotide binding	1286	429	357.56	2.6E-07
GO:0005085	guanyl-nucleotide exchange factor activity	71	40	19.74	2.6E-07
GO:0042302	structural constituent of cuticle	31	22	8.62	7.1E-07
GO:0003777	microtubule motor activity	22	16	6.12	1.5E-05
GO:0008536	Ran GTPase binding	13	11	3.61	3.3E-05
GO:0005509	calcium ion binding	192	78	53.38	7.5E-05
SG.UpLab.D4vsD0.CC					
GO:0016021	integral component of membrane	712	243	185.77	8.6E-08
GO:0005886	plasma membrane	99	50	25.83	0.00013
GO:0005911	cell-cell junction	6	6	1.57	0.00031
GO:0016020	membrane	1458	450	380.42	0.00077
GO:0031012	extracellular matrix	5	5	1.3	0.0012

GO:0005856	cytoskeleton	207	62	54.01	0.00231
GO:0016459	myosin complex	21	12	5.48	0.00244
GO:0045202	synapse	8	6	2.09	0.00529
GO:0030127	COPII vesicle coat	8	6	2.09	0.00529
GO:0000159	protein phosphatase type 2A complex	6	5	1.57	0.00564
SG.UpGlobal.D4vsD0.BP					
GO:0030682	mitigation of host defenses by symbiont	173	85	41.1	1.9E-13
GO:0006508	proteolysis	707	219	167.98	2.8E-08
GO:0006468	protein phosphorylation	459	122	109.05	5.7E-08
GO:0007155	cell adhesion	47	29	11.17	6.6E-07
GO:0009166	nucleotide catabolic process	63	32	14.97	2.7E-06
GO:0035556	intracellular signal transduction	387	95	91.95	1.3E-05
GO:0007165	signal transduction	678	207	161.09	1.4E-05
GO:0007010	cytoskeleton organization	147	52	34.93	7.7E-05
GO:0043547	positive regulation of GTPase activity	13	9	3.09	0.00065
GO:0044087	regulation of cellular component biogenesis	30	9	7.13	0.00076
SG.UpGlobal.D4vsD0.MF					
GO:0043176	amine binding	173	85	42.65	2E-12
GO:0004867	serine-type endopeptidase inhibitor activity	153	73	37.72	4.1E-10
GO:0008017	microtubule binding	51	32	12.57	8.6E-09

GO:0042302	structural constituent of cuticle	31	23	7.64	8.9E-09
GO:0000166	nucleotide binding	1286	354	317.04	1.8E-08
GO:0004222	metalloendopeptidase activity	219	91	53.99	2.1E-08
GO:0005524	ATP binding	802	250	197.72	7.8E-06
GO:0016788	hydrolase activity, acting on ester bonds	485	113	119.57	1.4E-05
GO:0004674	protein serine/threonine kinase activity	100	46	24.65	1.8E-05
GO:0005515	protein binding	2743	785	676.24	2.3E-05
SG.UpGlobal.D4vsD0.CC					
GO:0005886	plasma membrane	99	45	22.93	8.4E-05
GO:0016021	integral component of membrane	712	204	164.92	9.8E-05
GO:0031012	extracellular matrix	5	5	1.16	0.00066
GO:0005615	extracellular space	57	23	13.2	0.00264
GO:0005911	cell-cell junction	6	5	1.39	0.0032
GO:0016459	myosin complex	21	11	4.86	0.00334
GO:0016020	membrane	1458	390	337.72	0.00349
GO:0005643	nuclear pore	30	14	6.95	0.00382
GO:0043229	intracellular organelle	1855	382	429.67	0.00441
GO:0005856	cytoskeleton	207	53	47.95	0.00551
SG.DownWT.D4vsD0.BP					
GO:0030682	mitigation of host defenses by symbiont	173	54	28.37	7.8E-07
GO:0006260	DNA replication	92	27	15.09	0.00014
GO:0055114	oxidation-reduction process	650	128	106.61	0.0007

GO:0006364	rRNA processing	41	17	6.72	0.00103
GO:0006383	transcription by RNA polymerase III	15	8	2.46	0.00221
GO:0000027	ribosomal large subunit assembly	5	4	0.82	0.00313
GO:0006390	mitochondrial transcription	8	5	1.31	0.00426
GO:0106035	protein maturation by [4Fe-4S] cluster transfer	6	4	0.98	0.00817
GO:0001682	tRNA 5'-leader removal	7	4	1.15	0.01661
GO:0019509	L-methionine salvage from methylthioadenosine	7	4	1.15	0.01661
SG.DownWT.D4vsD0.MF					
GO:0005506	iron ion binding	190	60	30.69	4.6E-08
GO:0020037	heme binding	163	53	26.33	1.5E-07
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	194	55	31.33	4.4E-07
GO:0043176	amine binding	173	54	27.94	5.2E-07
GO:0004185	serine-type carboxypeptidase activity	32	14	5.17	0.0002
GO:0046982	protein heterodimerization activity	71	23	11.47	0.00053
GO:0004526	ribonuclease P activity	6	5	0.97	0.00057
GO:0008897	holo-[acyl-carrier-protein] synthase activity	5	4	0.81	0.00295
GO:0004109	coproporphyrinogen oxidase activity	5	4	0.81	0.00295

GO:0004104	cholinesterase activity	18	8	2.91	0.00434
SG.DownWT.D4vsD0.CC					
GO:0008180	COP9 signalosome	14	9	2.15	4.3E-05
GO:0000228	nuclear chromosome	64	18	9.81	4.6E-05
GO:0032993	protein-DNA complex	35	15	5.37	8E-05
GO:0005730	nucleolus	30	13	4.6	0.00022
GO:0000178	exosome (RNase complex)	13	7	1.99	0.00142
GO:0032299	ribonuclease H2 complex	5	4	0.77	0.00241
GO:0030914	STAGA complex	5	4	0.77	0.00241
GO:0005657	replication fork	5	4	0.77	0.00241
GO:0005681	spliceosomal complex	29	11	4.45	0.00248
GO:0030915	Smc5-Smc6 complex	11	6	1.69	0.00294
SG.DownLab.D4vsD0.BP					
GO:0046330	positive regulation of JNK cascade	140	52	25.95	1.3E-07
GO:0007250	activation of NF-kappaB-inducing kinase activity	140	52	25.95	1.3E-07
GO:0055114	oxidation-reduction process	650	162	120.49	9.4E-07
GO:0016226	iron-sulfur cluster assembly	17	12	3.15	3.9E-06
GO:0034220	ion transmembrane transport	144	37	26.69	1.9E-05
GO:0046081	dUTP catabolic process	6	6	1.11	4E-05
GO:0006226	dUMP biosynthetic process	6	6	1.11	4E-05
GO:0055070	copper ion homeostasis	8	6	1.48	0.0008
GO:0006352	DNA-templated transcription, initiation	52	23	9.64	0.0041
GO:0016070	RNA metabolic process	1144	229	212.06	0.0048

SG.DownLab.D4vsD0.MF					
GO:0005506	iron ion binding	190	82	34.92	4.4E-13
GO:0020037	heme binding	163	68	29.96	2.8E-12
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	194	71	35.66	6.3E-11
GO:0005230	extracellular ligand-gated ion channel activity	31	17	5.7	5.6E-06
GO:0046982	protein heterodimerization activity	71	29	13.05	8.4E-06
GO:0004170	dUTP diphosphatase activity	6	6	1.1	3.8E-05
GO:0005126	cytokine receptor binding	6	6	1.1	3.8E-05
GO:0008199	ferric iron binding	11	8	2.02	0.00012
GO:0004869	cysteine-type endopeptidase inhibitor activity	19	10	3.49	0.00078
GO:0008168	methyltransferase activity	214	58	39.34	0.00084
SG.DownLab.D4vsD0.CC					
GO:0016591	RNA polymerase II, holoenzyme	37	14	6.87	0.00071
GO:0030173	integral component of Golgi membrane	6	5	1.11	0.0011
GO:0008180	COP9 signalosome	14	8	2.6	0.00141
GO:0000178	exosome (RNase complex)	13	7	2.41	0.00446
GO:0032993	protein-DNA complex	35	14	6.49	0.0049
GO:0005730	nucleolus	30	12	5.57	0.00495
GO:0022624	proteasome accessory complex	9	4	1.67	0.00637

GO:0090575	RNA polymerase II transcription regulator complex	42	16	7.79	0.00728
GO:0005739	mitochondrion	260	68	48.25	0.01334
GO:0030880	RNA polymerase complex	49	20	9.09	0.02116
SG.DownGlobal.D4vsD0.BP					
GO:0055114	oxidation-reduction process	650	165	117.66	1.3E-08
GO:0046330	positive regulation of JNK cascade	140	50	25.34	4.4E-07
GO:0007250	activation of NF-kappaB-inducing kinase activity	140	50	25.34	4.4E-07
GO:0016226	iron-sulfur cluster assembly	17	12	3.08	3E-06
GO:0030682	mitigation of host defenses by symbiont	173	54	31.32	1.7E-05
GO:0034220	ion transmembrane transport	144	34	26.07	0.0003
GO:0006826	iron ion transport	8	6	1.45	0.0007
GO:0009143	nucleoside triphosphate catabolic process	12	9	2.17	0.00097
GO:0006260	DNA replication	92	25	16.65	0.0016
GO:0006383	transcription by RNA polymerase III	15	7	2.72	0.00375
SG.DownGlobal.D4vsD0.MF					
GO:0005506	iron ion binding	190	85	34.02	9E-15
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	194	72	34.74	1.9E-12
GO:0020037	heme binding	163	67	29.19	2.6E-12

GO:0004602	glutathione peroxidase activity	11	9	1.97	7.2E-06
GO:0008199	ferric iron binding	11	9	1.97	7.2E-06
GO:0043176	amine binding	173	54	30.98	1.3E-05
GO:0005126	cytokine receptor binding	6	6	1.07	3.3E-05
GO:0046982	protein heterodimerization activity	71	27	12.71	4.7E-05
GO:0004185	serine-type carboxypeptidase activity	32	15	5.73	0.00015
GO:0005230	extracellular ligand-gated ion channel activity	31	14	5.55	0.00041
SG.DownGlobal.D4vsD0.CC					
GO:0032993	protein-DNA complex	35	16	6.01	0.00014
GO:0005576	extracellular region	189	53	32.46	0.00021
GO:0005730	nucleolus	30	13	5.15	0.00069
GO:0030173	integral component of Golgi membrane	6	5	1.03	0.00076
GO:0090575	RNA polymerase II transcription regulator complex	42	16	7.21	0.0008
GO:0032299	ribonuclease H2 complex	5	4	0.86	0.00373
GO:0030914	STAGA complex	5	4	0.86	0.00373
GO:0005657	replication fork	5	4	0.86	0.00373
GO:0000228	nuclear chromosome	64	12	10.99	0.00429
GO:0008180	COP9 signalosome	14	7	2.4	0.00481

Table 3.5 – **Ten (max) most significantly enriched GO terms (lowest p-values) using goseq for each strain (WT, Lab) or regardless of the strain (global) for the D4 vs D0 comparison in each tissue (OV, MT, SG) independently of the ontology (BP, MF, CC)**
This table can be viewed at:
`https://drive.google.com/file/d/1FUcDoY2aLkQ-qevcAkPaIAoW71ldr2KO/view?usp=sharing`

Table 3.6 – **Ten (max) most significantly enriched GO terms (lowest p-values) using topGO for each time point (D4, D0) for the WT vs Lab comparison in each tissue (OV, MT, SG) for each ontology (BP, MF, CC)**
This table can be downloaded at: `https://drive.google.com/file/d/1sEPmsPJfEqtvaEVEdnk1NM9s_IRi7k0Y/view?usp=sharing`

GO.ID	Term	Annotated	Significant	Expected	weight01
OV.UpD4.WTvsLab.BP					
GO:0007250	activation of NF-kappaB-inducing kinase activity	140	6	1.29	0.0018
GO:0046330	positive regulation of JNK cascade	140	6	1.29	0.0018
GO:0097064	ncRNA export from nucleus	5	1	0.05	0.0454
GO:0006207	'de novo' pyrimidine nucleobase biosynthetic process	5	1	0.05	0.0454
GO:0046654	tetrahydrofolate biosynthetic process	5	1	0.05	0.0454
GO:0006420	arginyl-tRNA aminoacylation	5	1	0.05	0.0454
OV.UpD4.WTvsLab.MF					
GO:0004869	cysteine-type endopeptidase inhibitor activity	19	2	0.18	0.014
GO:0003899	DNA-directed 5'-3' RNA polymerase activity	30	2	0.29	0.033
GO:0016407	acetyltransferase activity	46	2	0.44	0.037
GO:0008270	zinc ion binding	568	10	5.4	0.043
GO:0004814	arginine-tRNA ligase activity	5	1	0.05	0.047
GO:0035596	methylthiotransferase activity	5	1	0.05	0.047

GO:0016417	S-acyltransferase activity	5	1	0.05	0.047
OV.UpD4.WTvsLab.CC					
GO:0042721	TIM22 mitochondrial import inner membrane insertion complex	8	2	0.07	0.002
GO:0005758	mitochondrial intermembrane space	18	2	0.16	0.01
GO:0016592	mediator complex	41	2	0.36	0.05
OV.UpD0.WTvsLab.BP					
GO:0055114	oxidation-reduction process	650	42	24.31	0.00017
GO:0051030	snRNA transport	6	3	0.22	0.00095
GO:0007586	digestion	14	4	0.52	0.00142
GO:0120009	intermembrane lipid transfer	5	2	0.19	0.01293
GO:0001522	pseudouridine synthesis	25	4	0.93	0.01302
GO:0055082	cellular chemical homeostasis	26	3	0.97	0.01889
GO:0046855	inositol phosphate dephosphorylation	6	2	0.22	0.01892
GO:0006284	base-excision repair	17	3	0.64	0.02385
GO:0033617	mitochondrial cytochrome c oxidase assembly	17	3	0.64	0.02385
GO:0006839	mitochondrial transport	41	4	1.53	0.03344
OV.UpD0.WTvsLab.MF					
GO:0005506	iron ion binding	190	21	7.61	2.3E-05
GO:0020037	heme binding	163	19	6.53	2.7E-05
GO:0004869	cysteine-type endopeptidase inhibitor activity	19	5	0.76	0.00073

GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	194	18	7.77	0.00078
GO:0004620	phospholipase activity	34	6	1.36	0.00457
GO:0009982	pseudouridine synthase activity	21	4	0.84	0.00882
GO:0120013	lipid transfer activity	5	2	0.2	0.01476
GO:0019200	carbohydrate kinase activity	16	3	0.64	0.0242
GO:0019904	protein domain specific binding	17	3	0.68	0.02853
GO:0099094	ligand-gated cation channel activity	7	2	0.28	0.02938
OV.UpD0.WTvsLab.CC					
GO:0005615	extracellular space	57	8	2.19	0.0013
GO:0042721	TIM22 mitochondrial import inner membrane insertion complex	8	3	0.31	0.0027
GO:0005576	extracellular region	189	18	7.25	0.0221
GO:0031966	mitochondrial membrane	127	12	4.87	0.0409
GO:0016459	myosin complex	21	3	0.81	0.0443
OV.DownD4.WTvsLab.BP					
GO:0042819	vitamin B6 biosynthetic process	5	1	0.03	0.028
GO:0070940	dephosphorylation of RNA polymerase II C-terminal domain	5	1	0.03	0.028
GO:0006379	mRNA cleavage	6	1	0.03	0.034
GO:0019722	calcium-mediated signaling	6	1	0.03	0.034
GO:0006370	7-methylguanosine mRNA capping	7	1	0.04	0.039
GO:0032509	endosome transport via multivesicular body sorting pathway	8	1	0.05	0.045

OV.DownD4.WTvsLab.MF					
GO:0003839	gamma-glutamylcyclotransferase activity	5	1	0.03	0.028
GO:0004109	coproporphyrinogen oxidase activity	5	1	0.03	0.028
GO:0047617	acyl-CoA hydrolase activity	5	1	0.03	0.028
GO:0008420	RNA polymerase II CTD heptapeptide repeat phosphatase activity	6	1	0.03	0.033
GO:0043130	ubiquitin binding	6	1	0.03	0.033
GO:0032266	phosphatidylinositol-3-phosphate binding	6	1	0.03	0.033
GO:0003747	translation release factor activity	8	1	0.04	0.044
GO:0008174	mRNA methyltransferase activity	8	1	0.04	0.044
GO:0016805	dipeptidase activity	8	1	0.04	0.044
GO:0004864	protein phosphatase inhibitor activity	8	1	0.04	0.044
OV.DownD4.WTvsLab.CC					
GO:0042025	host cell nucleus	139	3	0.7	0.031
GO:0042721	TIM22 mitochondrial import inner membrane insertion complex	8	1	0.04	0.04
GO:0031083	BLOC-1 complex	8	1	0.04	0.04
OV.DownD0.WTvsLab.BP					
GO:0046330	positive regulation of JNK cascade	140	9	2.53	0.00094
GO:0007250	activation of NF-kappaB-inducing kinase activity	140	9	2.53	0.00094

GO:0000387	spliceosomal snRNP assembly	23	3	0.42	0.00786
GO:0031365	N-terminal protein amino acid modification	8	2	0.14	0.00847
GO:0043066	negative regulation of apoptotic process	8	2	0.14	0.00847
GO:0055070	copper ion homeostasis	8	2	0.14	0.00847
GO:0007064	mitotic sister chromatid cohesion	9	2	0.16	0.01076
GO:0018342	protein prenylation	12	2	0.22	0.01903
GO:0009435	NAD biosynthetic process	14	2	0.25	0.02563
GO:0006914	autophagy	30	3	0.54	0.0366
OV.DownD0.WTvsLab.MF					
GO:0008897	holo-[acyl-carrier-protein] synthase activity	5	3	0.09	6.2E-05
GO:0004514	nicotinate-nucleotide diphosphorylase (carboxylating) activity	5	2	0.09	0.0033
GO:0008381	mechanosensitive ion channel activity	5	2	0.09	0.0033
GO:0047617	acyl-CoA hydrolase activity	5	2	0.09	0.0033
GO:0000287	magnesium ion binding	69	5	1.28	0.0091
GO:0004364	glutathione transferase activity	14	2	0.26	0.0271
GO:0019239	deaminase activity	15	2	0.28	0.0309
GO:0005507	copper ion binding	15	2	0.28	0.0309
GO:0003676	nucleic acid binding	1751	34	32.59	0.031
GO:0008168	methyltransferase activity	214	7	3.98	0.0416
OV.DownD0.WTvsLab.CC					

GO:0016021	integral component of membrane	712	30	15.73	0.00075
GO:0072546	ER membrane protein complex	7	2	0.15	0.00943
GO:0042721	TIM22 mitochondrial import inner membrane insertion complex	8	2	0.18	0.01239
MT.UpD4.WTvsLab.BP					
GO:0009405	pathogenesis	5	2	0.09	0.0029
GO:0051030	snRNA transport	6	2	0.1	0.0043
GO:0055114	oxidation-reduction process	650	20	11.32	0.0083
GO:0007009	plasma membrane organization	11	2	0.19	0.0149
GO:0015914	phospholipid transport	15	2	0.26	0.0272
GO:0006811	ion transport	306	8	5.33	0.0303
GO:0033617	mitochondrial cytochrome c oxidase assembly	17	2	0.3	0.0345
GO:0055085	transmembrane transport	582	14	10.13	0.0441
MT.UpD4.WTvsLab.MF					
GO:0020037	heme binding	163	9	2.92	0.0026
GO:0008200	ion channel inhibitor activity	5	2	0.09	0.0031
GO:0005506	iron ion binding	190	9	3.41	0.0072
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	194	10	3.48	0.0106
GO:0140303	intramembrane lipid transporter activity	10	2	0.18	0.0131
GO:0005548	phospholipid transporter activity	10	2	0.18	0.0131

GO:0004190	aspartic-type endopeptidase activity	28	3	0.5	0.0134
GO:0008270	zinc ion binding	568	17	10.19	0.0263
GO:0008061	chitin binding	65	4	1.17	0.029
MT.UpD4.WTvsLab.CC					
GO:0005576	extracellular region	189	9	3.77	0.014
MT.UpD0.WTvsLab.BP					
GO:0007186	G protein-coupled receptor signaling pathway	77	19	7.64	0.00014
GO:0007264	small GTPase mediated signal transduction	84	22	8.34	0.00018
GO:0032502	developmental process	88	22	8.74	0.00093
GO:0055085	transmembrane transport	582	79	57.78	0.00125
GO:0007165	signal transduction	678	98	67.31	0.00175
GO:0032870	cellular response to hormone stimulus	8	4	0.79	0.00486
GO:0006812	cation transport	197	27	19.56	0.00704
GO:0048513	animal organ development	9	4	0.89	0.00807
GO:0032784	regulation of DNA-templated transcription, elongation	10	3	0.99	0.00984
GO:0006694	steroid biosynthetic process	11	3	1.09	0.00984
MT.UpD0.WTvsLab.MF					
GO:0005085	guanyl-nucleotide exchange factor activity	71	23	7.64	6.9E-07

GO:0004114	3',5'-cyclic-nucleotide phosphodiesterase activity	10	5	1.08	0.0023
GO:0004930	G protein-coupled receptor activity	46	12	4.95	0.0027
GO:0008483	transaminase activity	23	7	2.48	0.0047
GO:0008083	growth factor activity	23	7	2.48	0.0085
GO:0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	83	14	8.93	0.0235
GO:0016879	ligase activity, forming carbon-nitrogen bonds	33	9	3.55	0.0291
GO:0099094	ligand-gated cation channel activity	7	3	0.75	0.0312
GO:0016888	endodeoxyribonuclease activity, producing 5'-phosphomonoesters	7	3	0.75	0.0312
GO:0045296	cadherin binding	7	3	0.75	0.0312
MT.UpD0.WTvsLab.CC					
GO:0016021	integral component of membrane	712	104	70.14	2.8E-06
GO:0016020	membrane	1458	193	143.63	0.00049
GO:0005887	integral component of plasma membrane	31	7	3.05	0.03067
MT.DownD4.WTvsLab.BP					
GO:0006627	protein processing involved in protein targeting to mitochondrion	8	2	0.08	0.0028
GO:0042819	vitamin B6 biosynthetic process	5	1	0.05	0.05

GO:0030512	negative regulation of transforming growth factor beta receptor signaling pathway	5	1	0.05	0.05
GO:0071569	protein ufmylation	5	1	0.05	0.05
GO:0007605	sensory perception of sound	5	1	0.05	0.05
MT.DownD4.WTvsLab.MF					
GO:0004523	RNA-DNA hybrid ribonuclease activity	19	3	0.18	0.00074
GO:0008270	zinc ion binding	568	12	5.45	0.008
GO:0008236	serine-type peptidase activity	233	5	2.24	0.01376
GO:0008146	sulfotransferase activity	115	5	1.1	0.02
GO:0003676	nucleic acid binding	1751	19	16.81	0.03423
GO:0005230	extracellular ligand-gated ion channel activity	31	2	0.3	0.0354
GO:0047617	acyl-CoA hydrolase activity	5	1	0.05	0.04708
GO:0004109	coproporphyrinogen oxidase activity	5	1	0.05	0.04708
GO:0015450	P-P-bond-hydrolysis-driven protein transmembrane transporter activity	5	1	0.05	0.04708
GO:0046332	SMAD binding	5	1	0.05	0.04708
MT.DownD4.WTvsLab.CC					
GO:0030880	RNA polymerase complex	49	2	0.5	0.039
GO:0005840	ribosome	357	8	3.61	0.05
MT.DownD0.WTvsLab.BP					

GO:0007250	activation of NF-kappaB-inducing kinase activity	140	37	7.88	5.9E-16
GO:0046330	positive regulation of JNK cascade	140	37	7.88	5.9E-16
GO:0006479	protein methylation	40	7	2.25	0.0003
GO:0043066	negative regulation of apoptotic process	8	4	0.45	0.00058
GO:0043281	regulation of cysteine-type endopeptidase activity involved in apoptotic process	5	3	0.28	0.00163
GO:1903046	meiotic cell cycle process	5	3	0.28	0.00163
GO:0006269	DNA replication, synthesis of RNA primer	6	3	0.34	0.00312
GO:0006275	regulation of DNA replication	6	3	0.34	0.00312
GO:0000725	recombinational repair	17	3	0.96	0.00316
GO:0006470	protein dephosphorylation	85	12	4.79	0.0039
MT.DownD0.WTvsLab.MF					
GO:0003993	acid phosphatase activity	15	5	0.89	0.0013
GO:0008897	holo-[acyl-carrier-protein] synthase activity	5	3	0.3	0.0019
GO:0004726	non-membrane spanning protein tyrosine phosphatase activity	11	4	0.65	0.0028
GO:0005126	cytokine receptor binding	6	3	0.35	0.0036
GO:0003676	nucleic acid binding	1751	128	103.38	0.004
GO:0016772	transferase activity, transferring phosphorus-containing groups	584	31	34.48	0.0101

GO:0008168	methyltransferase activity	214	20	12.63	0.0114
GO:0003700	DNA-binding transcription factor activity	206	20	12.16	0.0194
GO:0043565	sequence-specific DNA binding	129	15	7.62	0.0231
GO:0003697	single-stranded DNA binding	20	4	1.18	0.0274
MT.DownD0.WTvsLab.CC					
GO:0044815	DNA packaging complex	33	6	1.77	0.0081
GO:0000793	condensed chromosome	22	5	1.18	0.018
GO:0000776	kinetochore	5	2	0.27	0.0258
GO:0032039	integrator complex	13	3	0.7	0.0294
GO:0030677	ribonuclease P complex	6	2	0.32	0.0374
SG.UpD4.WTvsLab.BP					
GO:0030682	mitigation of host defenses by symbiont	173	44	4.49	<1e-30
GO:0009166	nucleotide catabolic process	63	11	1.64	5.2E-07
GO:0006508	proteolysis	707	38	18.36	9.5E-06
GO:0007586	digestion	14	3	0.36	0.0051
GO:0007009	plasma membrane organization	11	2	0.29	0.0316
SG.UpD4.WTvsLab.MF					
GO:0043176	amine binding	173	44	4.38	<1e-30
GO:0004867	serine-type endopeptidase inhibitor activity	153	30	3.87	9.5E-19
GO:0004252	serine-type endopeptidase activity	182	20	4.61	3.2E-08
GO:0004222	metalloendopeptidase activity	219	15	5.54	0.00045
GO:0008237	metallopeptidase activity	304	23	7.69	0.00106

GO:0016717	oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	5	2	0.13	0.00606
GO:0008241	peptidyl-dipeptidase activity	9	2	0.23	0.02042
GO:0140303	intramembrane lipid transporter activity	10	2	0.25	0.0251
GO:0005548	phospholipid transporter activity	10	2	0.25	0.0251
GO:0000166	nucleotide binding	1286	16	32.54	0.04097
SG.UpD4.WTvsLab.CC					
GO:0030173	integral component of Golgi membrane	6	2	0.09	0.0034
GO:0005615	extracellular space	57	4	0.88	0.011
GO:0005576	extracellular region	189	10	2.92	0.0341
SG.UpD0.WTvsLab.BP					
GO:0009166	nucleotide catabolic process	63	18	6.61	5.3E-05
GO:0006508	proteolysis	707	100	74.23	0.0013
GO:0051030	snRNA transport	6	4	0.63	0.0015
GO:0030682	mitigation of host defenses by symbiont	173	29	18.16	0.0071
GO:0007030	Golgi organization	5	3	0.52	0.0098
GO:0097064	ncRNA export from nucleus	5	3	0.52	0.0098
GO:0006096	glycolytic process	16	5	1.68	0.0205
GO:0007010	cytoskeleton organization	147	19	15.43	0.0217

GO:0007018	microtubule-based movement	36	8	3.78	0.0277
GO:0043087	regulation of GTPase activity	25	6	2.62	0.0299
SG.UpD0.WTvsLab.MF					
GO:0000166	nucleotide binding	1286	159	132.78	0.00031
GO:0003860	3-hydroxyisobutyryl-CoA hydrolase activity	5	4	0.52	0.00052
GO:0016788	hydrolase activity, acting on ester bonds	485	57	50.08	0.0033
GO:0004252	serine-type endopeptidase activity	182	31	18.79	0.00348
GO:0005524	ATP binding	802	106	82.8	0.00386
GO:0016887	ATPase activity	142	23	14.66	0.00428
GO:0008017	microtubule binding	51	12	5.27	0.00486
GO:0003777	microtubule motor activity	22	7	2.27	0.00519
GO:0004177	aminopeptidase activity	17	6	1.76	0.00543
GO:0030145	manganese ion binding	17	6	1.76	0.00543
SG.UpD0.WTvsLab.CC					
GO:0005783	endoplasmic reticulum	73	15	7.04	0.0088
GO:0005819	spindle	15	4	1.45	0.0231
GO:0030126	COPI vesicle coat	7	3	0.67	0.0231
GO:0032300	mismatch repair complex	7	3	0.67	0.0231
GO:0032039	integrator complex	13	4	1.25	0.03
GO:0042721	TIM22 mitochondrial import inner membrane insertion complex	8	3	0.77	0.0344
GO:0016021	integral component of membrane	712	81	68.62	0.0412
GO:0000124	SAGA complex	15	4	1.45	0.0492

SG.DownD4.WTvsLab.BP					
GO:0055085	transmembrane transport	582	54	20.18	8.2E-14
GO:0006508	proteolysis	707	49	24.52	1.6E-08
GO:0006548	histidine catabolic process	5	4	0.17	6.9E-06
GO:0009166	nucleotide catabolic process	63	10	2.18	5.3E-05
GO:0006536	glutamate metabolic process	6	3	0.21	0.00076
GO:0043603	cellular amide metabolic process	542	14	18.8	0.00115
GO:0030682	mitigation of host defenses by symbiont	173	14	6	0.00266
GO:0044282	small molecule catabolic process	59	10	2.05	0.00591
GO:0035735	intraciliary transport involved in cilium assembly	5	2	0.17	0.01118
GO:0006189	'de novo' IMP biosynthetic process	5	2	0.17	0.01118
SG.DownD4.WTvsLab.MF					
GO:0022857	transmembrane transporter activity	614	58	22.74	2.4E-21
GO:0004867	serine-type endopeptidase inhibitor activity	153	33	5.67	9E-17
GO:0004222	metalloendopeptidase activity	219	28	8.11	8.8E-09
GO:0008146	sulfotransferase activity	115	19	4.26	8.5E-08
GO:0008061	chitin binding	65	13	2.41	5.7E-07
GO:0016812	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amides	5	3	0.19	0.00048
GO:0003824	catalytic activity	4621	203	171.13	0.0009
GO:0016787	hydrolase activity	1952	105	72.29	0.00096

GO:0004252	serine-type endopeptidase activity	182	15	6.74	0.00311
GO:0043176	amine binding	173	14	6.41	0.00496
SG.DownD4.WTvsLab.CC					
GO:0016021	integral component of membrane	712	38	18.77	3.3E-06
GO:0005576	extracellular region	189	11	4.98	0.01
GO:0005868	cytoplasmic dynein complex	10	2	0.26	0.027
SG.DownD0.WTvsLab.BP					
GO:0007250	activation of NF-kappaB-inducing kinase activity	140	26	9.69	2.8E-06
GO:0046330	positive regulation of JNK cascade	140	26	9.69	2.8E-06
GO:0009253	peptidoglycan catabolic process	20	9	1.38	2.9E-06
GO:0055114	oxidation-reduction process	650	72	45	3E-06
GO:0006548	histidine catabolic process	5	4	0.35	0.00011
GO:0034220	ion transmembrane transport	144	14	9.97	0.00014
GO:0055085	transmembrane transport	582	62	40.29	0.00099
GO:0042073	intraciliary transport	9	4	0.62	0.00125
GO:0006826	iron ion transport	8	4	0.55	0.00127
GO:0006412	translation	478	49	33.09	0.00134
SG.DownD0.WTvsLab.MF					
GO:0008146	sulfotransferase activity	115	31	8.36	9.5E-11
GO:0005506	iron ion binding	190	38	13.81	1.6E-07
GO:0008061	chitin binding	65	18	4.73	5.1E-07
GO:0020037	heme binding	163	31	11.85	5.9E-07

GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	194	34	14.11	2.9E-06
GO:0008745	N-acetylmuramoyl-L-alanine amidase activity	20	9	1.45	4.4E-06
GO:0022857	transmembrane transporter activity	614	70	44.64	0.0001
GO:0033735	aspartate dehydrogenase activity	5	4	0.36	0.00013
GO:0005230	extracellular ligand-gated ion channel activity	31	9	2.25	0.00025
GO:0004364	glutathione transferase activity	14	6	1.02	0.00026
SG.DownD0.WTvsLab.CC					
GO:0030286	dynein complex	29	8	1.89	0.00012
GO:0005840	ribosome	357	43	23.29	0.00026
GO:0005576	extracellular region	189	24	12.33	0.00027
GO:0030990	intraciliary transport particle	6	3	0.39	0.00473
GO:0015935	small ribosomal subunit	33	6	2.15	0.01807
GO:0005750	mitochondrial respiratory chain complex III	5	2	0.33	0.03716

Table 3.7 – **Ten (max) most significantly enriched GO terms (lowest p-values) using goseq for each time point (D4, D0) for the WT vs Lab comparison in each tissue (OV, MT, SG) independently of the ontology (BP, MF, CC)**
This table can be viewed at:
`https://drive.google.com/file/d/12poZmessBN-fp65QrXy8y0AxY64ESNgS/view?usp=sharing`

Additional figures, tables and material

Supplementary files

File SF1 - Trinotate report
`https://drive.google.com/file/d/18oiF-YOu-n_VEQWHac8aVEnM1LRtCrQ3/view?usp=sharing`

File SF2 - Tables of romer results for each strain (WT, Lab) or regardless the strain (global) for the D4 vs D0 comparison in each tissue (OV, MT, SG) independently of the ontology
`https://drive.google.com/file/d/1h3aPhKkriMBWifruTeBFzCaXzLg3pqGM/view?usp=sharing`

File SF3 - Tables of camera results for each strain (WT, Lab) or regardless the strain (global) for the D4 vs D0 comparison in each tissue (OV, MT, SG) independently of the ontology
`https://drive.google.com/file/d/14-6Bk3wVNxf5DO63ZHYIFQ26rma9M_80/view?usp=sharing`

File SF4 - Tables of romer results for each time point (D4, D0) or regardless the time point (global) for the WT vs Lab comparison in each tissue (OV, MT, SG) independently of the ontology
`https://drive.google.com/file/d/1kvaC3caAd7Xsk3dQmYdrjgI2fDVmj04R/view?usp=sharing`

SF5 - Tables of camera results for each time point (D4, D0) or regardless the time point (global) for the WT vs Lab comparison in each tissue (OV, MT, SG) independently

of the ontology
`https://drive.google.com/file/d/1mxYmoAYkZ9h603Ce2hstKPE3QS1G2yCj/view?usp=sharing`

Supplementary figures

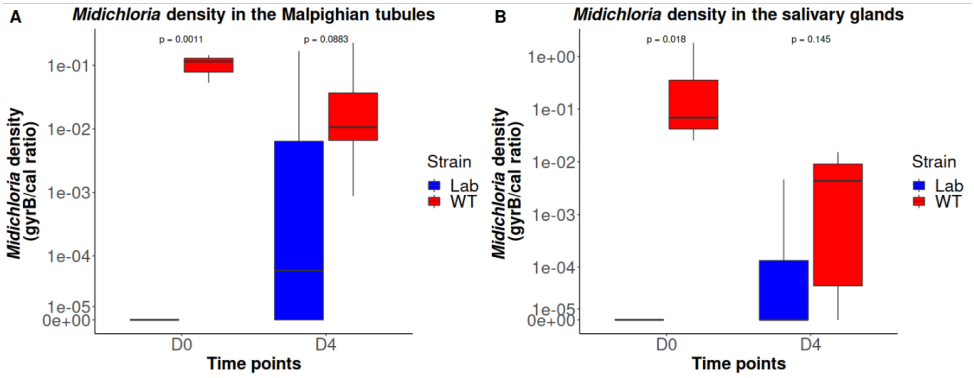

Figure S1 – ***Midichloria mitochondrii* density in (A) the Malpighian tubules and (B) salivary glands, at D0 and D4, in WT and Lab strains.**
The scale is a pseudo-log transformation with sigma = 1e-05, in order to avoid infinite values.

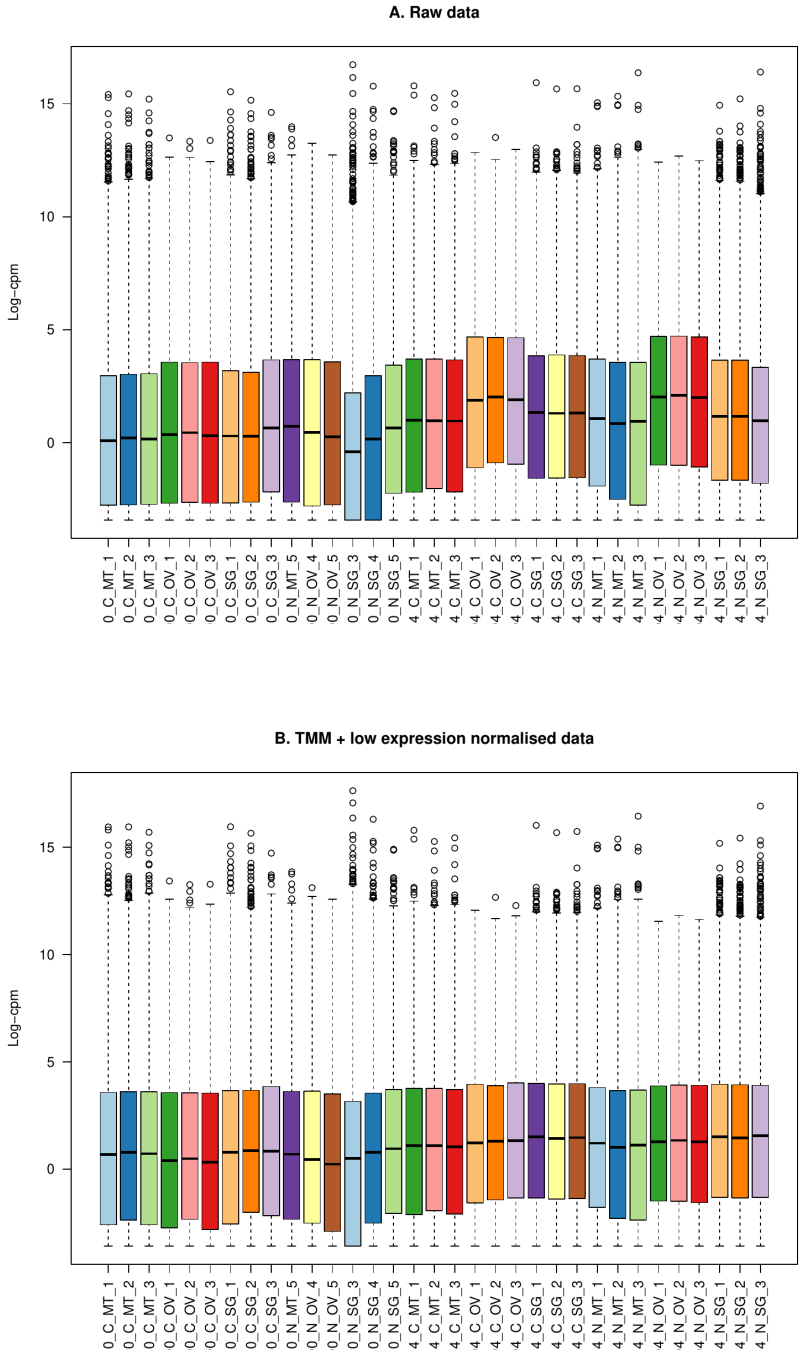

Figure S2 – **Boxplots of log-CPM values showing the expression distributions** for unnormalised data (A) and TMM and low expression normalised data (B) for each sample.

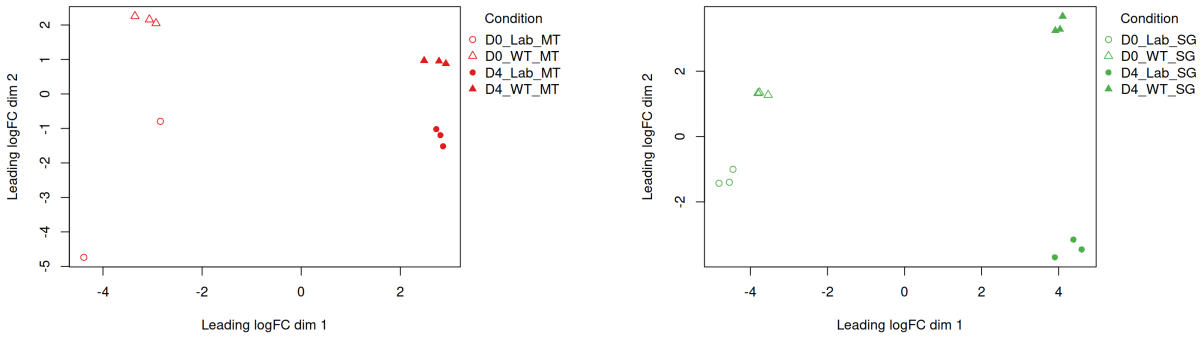

Figure S3 – **MDS plots for (A) the Malpighian tubules (red) and (B) the salivary glands (green)**

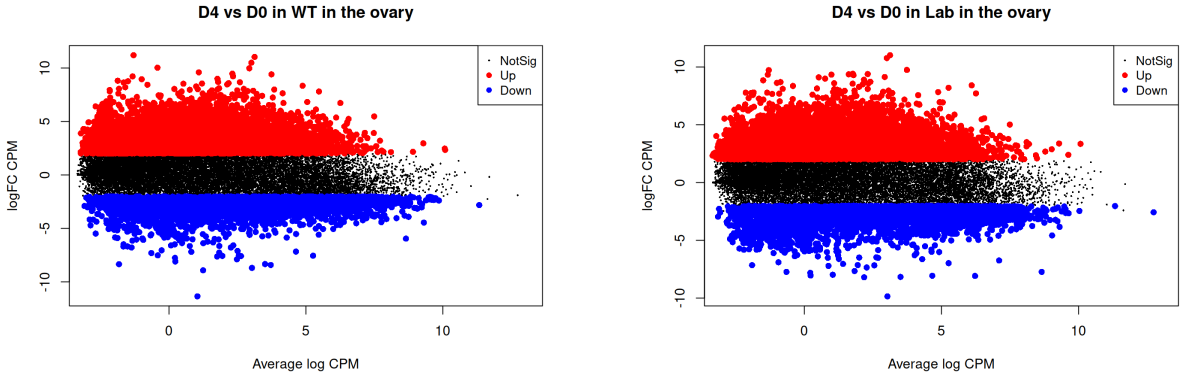

Figure S4 – **MD plots for the ovary showing genes that are differentially expressed within D4 vs D0 comparison** in WT (left) and in Lab (right). Each dot represents a gene. A red dot means that the gene is up-regulated in D4 while a blue dot means that the gene is down-regulated in D4. Black dots stand for non differentially expressed genes. Exact numbers of differentially expressed genes can be found in Table 3.2.

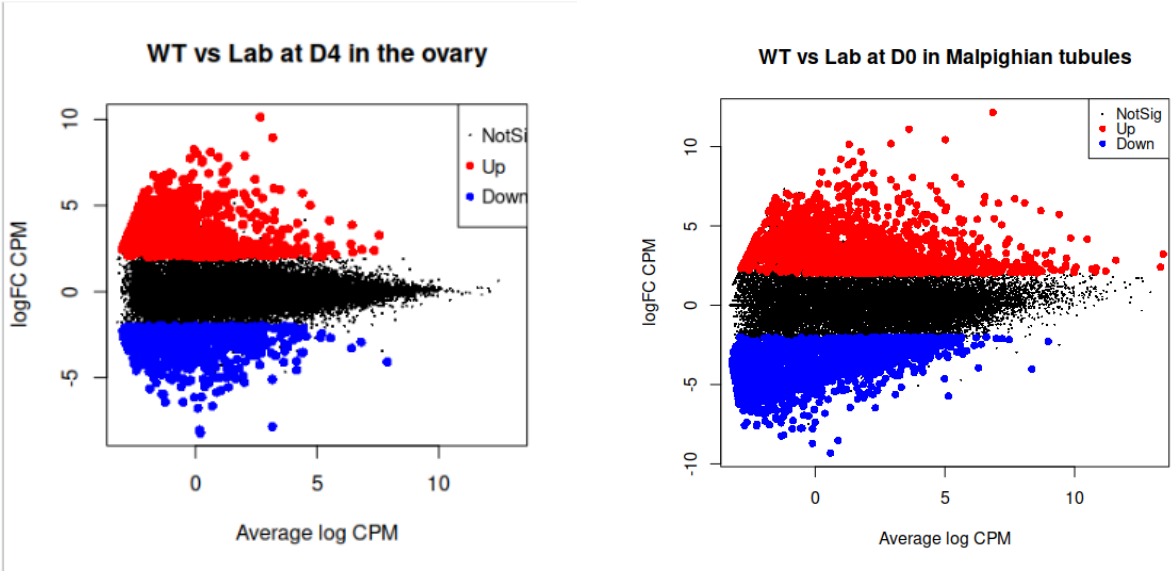

Figure S5 – **MD plots for the ovary showing genes that are differentially expressed within the WTvsLab comparison**, at D4 (left) and at D0 (right). Each dot represents a gene. A red dot means that the gene is up-regulated in D4 while a blue dot means that the gene is down-regulated in D4. Black dots stand for non differentially expressed genes. Exact numbers of differentially expressed genes can be found in Table 3.3.

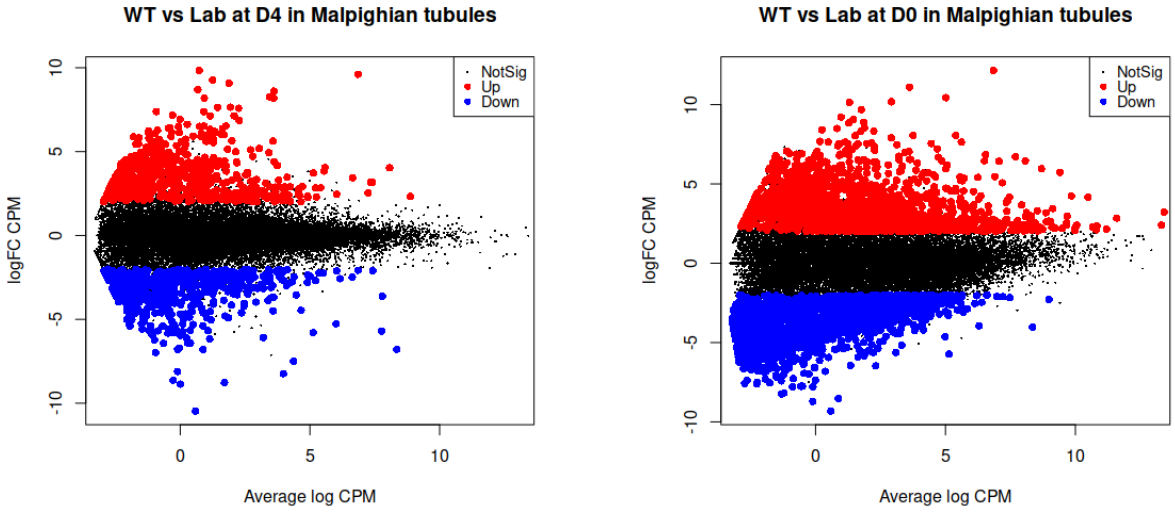

Figure S6 – **MD plots for the Malpighian tubules showing genes that are differentially expressed within the WTvsLab comparison**, at D4 (left) and at D0 (right). Each dot represents a gene. A red dot means that the gene is up-regulated in D4 while a blue dot means that the gene is down-regulated in D4. Black dots stand for non differentially expressed genes. Exact numbers of differentially expressed genes can be found in Table 3.3.

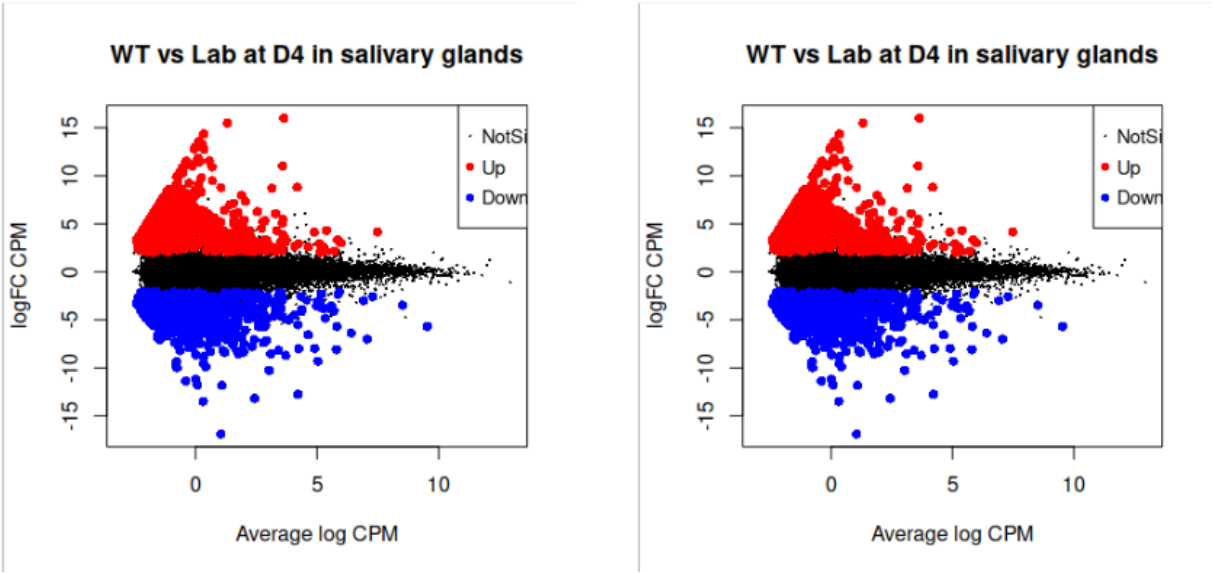

Figure S7 – **MD plots for the Malpighian tubules showing genes that are differentially expressed within the WTvsLab comparison**, at D4 (left) and at D0 (right). Each dot represents a gene. A red dot means that the gene is up-regulated in D4 while a blue dot means that the gene is down-regulated in D4. Black dots stand for non differentially expressed genes. Exact numbers of differentially expressed genes can be found in Table 3.3.

Supplementary tables

Table S1 – **Sample information, regarding feeding status, strain and tissue.**
A complete overview of the table can be found at:
`https://drive.google.com/file/d/11L477DaxJSazwY-i_z-cLUSWNp0Dd2mD/view?usp=sharing`

SRA accession number	File name	Feeding status	Strain	Tissue	GPS coordinates	Raw reads	Trimmed reads		Reads mapped to transcriptome	
						Number	Number	Percentage	Number	Percentage
SRR12606749	D0_C_MT_1	Unfed	WT	Malpighian Tubules	46°08'49.0"N 0°25'30.6"W	47,836,740	41,776,860	87.33	36,012,914	86.20
SRR12606748	D0_C_MT_2	Unfed	WT	Malpighian Tubules	46°08'49.0"N 0°25'30.6"W	46,756,034	40,842,584	87.35	35,662,278	87.32
SRR12606737	D0_C_MT_3	Unfed	WT	Malpighian Tubules	46°08'49.0"N 0°25'30.6"W	44,848,550	39,352,094	87.74	33,510,010	85.15
SRR12606726	D0_C_OV_1	Unfed	WT	Ovary	46°08'49.0"N 0°25'30.6"W	43,971,460	38,586,976	87.75	30,291,410	78.50
SRR12606720	D0_C_OV_2	Unfed	WT	Ovary	46°08'49.0"N 0°25'30.6"W	43,949,490	38,034,442	86.54	28,980,509	76.20
SRR12606719	D0_C_OV_3	Unfed	Lab	Ovary	46°08'49.0"N 0°25'30.6"W	42,629,066	38,013,196	89.17	30,105,122	79.20
SRR12606718	D0_C_SG_1	Unfed	Lab	Salivary Glands	46°08'49.0"N 0°25'30.6"W	44,348,888	36,338,214	81.94	31,249,249	86.00
SRR12606717	D0_C_SG_2	Unfed	Lab	Salivary Glands	46°08'49.0"N 0°25'30.6"W	46,519,188	39,988,758	85.96	36,506,182	91.29
SRR12606716	D0_C_SG_3	Unfed	Lab	Salivary Glands	46°08'49.0"N 0°25'30.6"W	41,274,068	35,860,162	86.88	30,585,179	85.29
SRR12606715	D0_N_MT_4	Unfed	Lab	Malpighian Tubules	46°59'60.0"N 6°56'58.7"E	45,491,268	40,292,080	88.57	34,621,493	85.93
SRR12606747	D0_N_MT_5	Unfed	Lab	Malpighian Tubules	46°59'60.0"N 6°56'58.7"E	41,130,294	36,051,684	87.65	29,721,033	82.44
SRR12606746	D0_N_OV_3	Unfed	Lab	Ovary	46°59'60.0"N 6°56'58.7"E	46,924,468	36,734,888	78.29	33,345,515	90.77
SRR12606745	D0_N_OV_4	Unfed	Lab	Ovary	46°59'60.0"N 6°56'58.7"E	48,403,244	44,163,544	91.24	36,924,333	83.61
SRR12606744	D0_N_OV_5	Unfed	Lab	Ovary	46°59'60.0"N 6°56'58.7"E	44,077,444	39,927,152	90.58	33,326,624	83.47
SRR12606743	D0_N_SG_3	Unfed	Lab	Salivary Glands	46°59'60.0"N 6°56'58.7"E	43,277,856	39,725,756	91.79	31,797,188	80.04
SRR12606742	D0_N_SG_4	Unfed	Lab	Salivary Glands	46°59'60.0"N 6°56'58.7"E	45,711,110	36,279,008	79.37	34,368,123	94.73
SRR12606741	D0_N_SG_5	Unfed	Lab	Salivary Glands	46°59'60.0"N 6°56'58.7"E	46,462,224	38,870,932	83.66	36,087,495	92.84
SRR12606740	D4_C_MT_1	Partially fed	WT	Malpighian Tubules	46°08'49.0"N 0°25'30.6"W	48,866,028	47,784,526	97.79	37,834,438	79.18
SRR12606739	D4_C_MT_2	Partially fed	WT	Malpighian Tubules	46°08'49.0"N 0°25'30.6"W	79,394,186	77,307,160	97.37	59,668,967	77.18
SRR12606738	D4_C_MT_3	Partially fed	WT	Malpighian Tubules	46°08'49.0"N 0°25'30.6"W	67,127,972	65,154,526	97.06	52,569,334	80.68
SRR12606736	D4_C_OV_1	Partially fed	WT	Ovary	46°08'49.0"N 0°25'30.6"W	74,258,186	72,363,014	97.45	51,910,446	71.74
SRR12606735	D4_C_OV_2	Partially fed	WT	Ovary	46°08'49.0"N 0°25'30.6"W	57,129,220	55,530,818	97.20	40,076,249	72.17
SRR12606734	D4_C_OV_3	Partially fed	WT	Ovary	46°08'49.0"N 0°25'30.6"W	64,981,958	63,363,416	97.51	45,310,482	71.51
SRR12606733	D4_C_SG_1	Partially fed	WT	Salivary Glands	46°08'49.0"N 0°25'30.6"W	52,113,568	50,924,980	97.72	40,781,991	80.08
SRR12606732	D4_C_SG_2	Partially fed	WT	Salivary Glands	46°08'49.0"N 0°25'30.6"W	52,280,396	50,907,094	97.37	39,621,976	77.83
SRR12606731	D4_C_SG_3	Partially fed	WT	Salivary Glands	46°08'49.0"N 0°25'30.6"W	61,815,342	60,246,828	97.46	47,325,164	78.55
SRR12606730	D4_N_MT_1	Partially fed	Lab	Malpighian Tubules	46°59'60.0"N 6°56'58.7"E	84,942,106	82,601,568	97.24	65,226,578	78.97
SRR12606729	D4_N_MT_2	Partially fed	Lab	Malpighian Tubules	46°59'60.0"N 6°56'58.7"E	56,693,356	55,076,698	97.15	45,668,074	82.92
SRR12606728	D4_N_MT_3	Partially fed	Lab	Malpighian Tubules	46°59'60.0"N 6°56'58.7"E	46,720,832	45,423,962	97.22	37,406,144	82.35
SRR12606727	D4_N_OV_1	Partially fed	Lab	Ovary	46°59'60.0"N 6°56'58.7"E	78,445,136	76,244,554	97.19	56,964,013	74.71
SRR12606725	D4_N_OV_2	Partially fed	Lab	Ovary	46°59'60.0"N 6°56'58.7"E	73,820,618	71,999,648	97.53	52,090,022	72.35
SRR12606724	D4_N_OV_3	Partially fed	Lab	Ovary	46°59'60.0"N 6°56'58.7"E	87,928,994	85,518,000	97.26	63,045,287	73.72
SRR12606723	D4_N_SG_1	Partially fed	Lab	Salivary Glands	46°59'60.0"N 6°56'58.7"E	56,297,918	54,834,560	97.40	45,390,667	82.78
SRR12606722	D4_N_SG_2	Partially fed	Lab	Salivary Glands	46°59'60.0"N 6°56'58.7"E	55,994,990	54,622,386	97.55	45,966,960	84.15
SRR12606721	D4_N_SG_3	Partially fed	Lab	Salivary Glands	46°59'60.0"N 6°56'58.7"E	50,376,974	48,932,256	97.13	43,432,365	88.76
	Total					1,912,799,172	1,779,674,324	92.13	1,433,383,814	81.67
	Min					41,130,294	35,860,162	78.29	28,980,509	71.51
	Max					87,928,994	85,518,000	97.79	65,226,578	94.73
	Mean					54,651,405	50,847,838	92.13	40,953,823	81.67

Table S2 – **Contig number and peptide prediction metrics of the the meta-transcriptome.**

Metrics		Number
Number of contigs		36,499
Number of peptides predicted (%)		47,822 (100)
	Complete	32,257 (67.45)
	3-prime partial	3,663 (7.66)
	5-prime partial	10,140 (21.20)
	Internal	1,762 (3.69)

GENERAL DISCUSSION

There is a growing concern for ticks and the threat that they represent for human and animal health through the vectorization of pathogens leading to disease development. This concern is even exacerbated by the unknown consequences of climate change on vector-borne diseases (Medlock and Leach, 2015). Among obligate hematophagous arthropods (Rio et al., 2016) or more generally arthropods with a very narrow diet based on the consumption of a nutrient fluid provided by their host such as developed sap for sap-feeding insects (Baumann et al., 1995), an increasing number of studies illustrates that such arthropods are only able to survive thanks to the presence of bacteria that are obligate symbionts, for instance by providing essential amino-acids that they do not find in their diet (Moran et al., 2008). Although they are partly responsible for the "ecological and evolutionary success" of those arthropods, such an obligate dependency to those particular microorganisms could also be considered as their weakness or Achilles heel (Bennett and Moran, 2015). Thus, one way to potentially exploit tick weaknesses and therefore to improve their control is to understand the subtleties of the interplays between ticks and their microbiome, in particular their main endosymbionts (in terms of prevalence and relative density compared to the rest of the microbiome; (Ben-Yosef et al., 2020; Chicana et al., 2019; Duron et al., 2018; Guizzo et al., 2020; Swei and Kwan, 2017). Removal of obligate symbionts and so microbiome manipulation is a strategy that has already been used to control some parasites, for instance some filarial nematodes such as *Onchocerca volvulus* or *Brugia malayi* targeting the obligate symbiont *Wolbachia* by the use of antibiotics (Hoerauf et al., 2001; Hoerauf et al., 2000; Hoerauf, 2008; Ru, 2005). Moreover, endosymbionts may be involved in the vector capacities of their arthropod host (Zindel et al., 2011). While this topic is receiving attention from researchers for more than a decade in the case of mosquitoes (Iturbe-Ormaetxe et al., 2011; Ye et al., 2013), this aspect has been poorly investigated for ticks until now (but see Budachetri et al., 2018). Paratransgenesis, a method which attempts to eliminate a pathogen from vector populations through transgenesis of a vector symbiont, is also receiving a growing interest for the control of vector-borne disease (Miller, 2011). Besides the role of some obligate symbionts in the nutrition of their arthropod host, endosymbionts may also be involved

in the manipulation of their host reproduction (Nguyen et al., 2017; Perlman et al., 2008; Werren et al., 2008) or in symbiont mediated protection (Brownlie and Johnson, 2009; Haine, 2008). However, none of such phenotypic changes have been investigated to our knowledge in the case of ticks. Thus, whatever are the consequences of endosymbionts on their arthropod host, a better understanding of the interplay with their host would be highly valuable to pave the way for control of ticks and tick-borne diseases.

This thesis has the purpose to better characterize the role of *Midichloria mitochondrii* on the biology of the hard tick *Ixodes ricinus* the most prevalent tick species in Europe. In the previous main chapters, I have evoked the following points: (i) the *M. mitochondrii* dynamics at the nymphal stage, (ii) the investigation of several tick life-history traits following the injection of antibiotics in order to remove *M. mitochondrii* from the tick or supplementation of B vitamins and (iii) the gene expression of three *I. ricinus* organs at different time points given the presence or absence of *M. mitochondrii*. This section is complementary to the discussions of the previous chapters, with the idea in mind to focus on the *M. mitochondrii–I. ricinus* interactions.

What knowledge is provided about the interplay between *Midichloria mitochondrii* and *Ixodes ricinus* through the investigation of the symbiont dynamics in nymphs?

Because of its maternally inherited feature, *M. mitochondrii* is present in its arthropod host since the very first step of embryogenesis, from the first egg stage to sexually immature stages (larvae and nymphs) and finally to adults. Even if most of the investigations to date about the couple *M. mitochondrii–I. ricinus* has been conducted on adults females, exploring the interplay between *M. mitochondrii* and its arthropod host during sexually immature stages is informative to understand the dialogue between the endosymbiont and the tick. The first chapter emphasizes a likely specialization of *M. mitochondrii* towards females already at the nymphal stage. At this stage, the ovary primordia start to develop and specialize to accomplish their future role at the adult stage regarding oogenesis and the development of the progeny. All the different stages and both sexes require vitamins

and co-factors not found in the blood for their proper development. We could make the hypothesis that if *M. mitochondrii* is completely absent early in nymphs that will become male, even if the male energetic needs are limited as revealed by the absence of blood meal for this sex at the adult stage, the endosymbiont is likely not a B vitamin provider (or does not assume this role alone). We found that *M. mitochondrii* is not strictly absent in male-to-be nymphs, but is rather gradually lost during ontogenesis. Therefore, those observations are not sufficient alone to prove that *M. mitochondrii* is a B vitamin provider. However, because some vitamins are needed in relatively limited quantities (other vitamins being used as precursors; Douglas, 2017), we cannot refute that the vitamins found in the eggs, provided by *M. mitochondrii* following the maternal transmission of bacteria, could be sufficient for larval or even nymphal development, in the case *M. mitochondrii* is the sole B vitamin provider.

The hypothesis of the potential role involving the bacteria during the moulting process (Zchori-Fein and Bourtzis, 2011) is somehow counter-intuitive given the disparity of the repartition of the symbionts in male and female nymphs. Indeed, if *M. mitochondrii* was supposed to help the tick in some manner to moult and that this role is density dependent, we would not have observed such a difference in symbiont density in the engorged nymphs of the two sexes. *Midichloria mitochondrii* characterization at the larval stage according to the tick sex could bring further information concerning the stage during the tick life-cycle when the endosymbiont is "lost" by the tick, or at least when the difference in density appears between males and females. However, new molecular tools to determine the sex of larvae are needed to tackle this question. The different ongoing genomic projects on several tick genomes should soon provide such useful tools (Gulia-Nuss et al., 2016; Jia et al., 2020; Murgia et al., 2019). As males have test testes instead of ovary and a reduced density of *M. mitochondrii* compared to females, the reduced density of the symbiont could be explained by its presence in Malpighian tubules (Olivieri et al., 2019). Such marked symbiont density between male and females inside Malpighian tubules happens to be the case for some other tick-symbiont associations, but is slightly different compared to the *M. mitochondrii–I. ricinus* association, as adult males harbour the symbiont at a much higher prevalence and relative density (Ben-Yosef et al., 2020; Lalzar et al., 2014; Lalzar et al., 2012). However, this point has not been investigated yet, and due to the low density of the symbionts in Malpighian tubules at the adult stage of *I. ricinus*, even in engorged females, it would be difficult to provide conclusive evidence on this topic

regarding the immature stages (as those organs are less developed and more challenging, if not impossible, to dissect).
The proximal reasons behind the lack of development of *M. mitochondrii* in the tick testes are still unknown. However, from an evolutionary point of view, testes are a dead-end for most maternally transmitted bacteria. This provides a likely explanation for the mechanism preventing symbiont multiplication in males (which would correspond to a metabolic cost). In addition, the moment at the immature stage when *M. mitochondrii* invades the female gonad tissues, starts to proliferate and to specialize towards a dedicated organ (the ovary) is still a matter of speculation, even if this is presumably tightly related with the development of this organ that can be considered as a receptacle for the endosymbiont.
The differences of *M. mitochondrii* titers observed within the different organs at the adult stage allows us to wonder if an "active migration" of the bacteria to reach those different tissues is responsible for this contrasted pattern (which would exhibit differential tropisms) or if a differential multiplication or survival occurs in them. The former question related to the symbiont migration towards different tissues may also have important consequences for pathogen transmission to vertebrates during the blood meal, if such an organ tropism is observed for salivary glands (Cafiso et al., 2018; Mariconti et al., 2012). Again, our observations do not allow us to distinguish those two alternative (but not exclusive) hypotheses. The ability to distinguish germ from somatic lines early during tick morphogenesis could help to test such hypotheses. Investigations on tick hematocytes and tick hemolymph could also provide interesting information on this topic. Despite the technical challenge, the visualization of *M. mitochondrii* (for example after GFP tagging) during tissue development would be especially interesting to follow their "spread" (localization) and their multiplication. The small size of larvae (and even nymphs) allows the visualization of several internal organs within the tick body without dissection by transparency through the thin cuticle, before the melanization (Fig. 13).

Although not investigated here, the regulation of symbiont titer (here between males and females) could also involve the immune system of the host (López-Madrigal and Duarte, 2019). In the case of *I. ricinus*, as adult males usually do not take a blood meal, those mechanisms could be very different between the two sexes.
Finally, it is worth highlighting that those different non-exclusive hypotheses on the consequences of *M. mitochondrii* density on its host metabolism, physiology or reproduction (here by comparing nymphs of the two sexes, before and after engorgement) illustrate

Figure 13 – ***Ixodes accuminatus* larva**
Note the transparency of the cuticle letting the visualization of some internal organs (Picture: O. PLANTARD)

that all the mentioned biological processes are often interdependent and involve different organs (such as gonads, Malpighian tubules or salivary glands) with different dynamics of genes expression according to the sex, stage and feeding status.

Injection of tetracycline antibiotics in the attempt to remove *Midichloria mitochondrii* and supplementation of B vitamins

Since the first premises of Bandi's and Sassera's labs regarding the formal characterization of *M. mitochondrii*, notably the discovery of its peculiar non-mandatory intramitochondrial tropism, the almost 100% prevalence in wild *I. ricinus* eggs, larvae and female adults, the intermediate and high prevalence in other tick species or the genome sequencing of the endosymbiont, the question of the role of *M. mitochondrii* on *I. ricinus* remains. Due to the reasons already mentioned in Chapter II, no conclusion could be drawn regarding the provision of B vitamins by *M. mitochondrii* to its host. Nonetheless, in this section, the possible involvement of *M. mitochondrii* in this provision is discussed.

Can *M. mitochondrii* be a B vitamin provider?

The availability of an increasing number of genomes of obligate endosymbionts associated with ticks (Duron et al., 2018; Gerhart et al., 2018; Gerhart et al., 2016; Gillespie et al., 2012; Gottlieb et al., 2015; Kurtti et al., 2015; Nardi et al., 2021; Sassera et al., 2011; Smith et al., 2015) shows a nutritional convergence regarding the provisioning of several vitamins of the B group (Duron and Gottlieb, 2020). *Coxiella*-like endosymbiont (CLE) and *Francisella*-like endosymbiont (FLE) notably possess the entire pathways for the biosynthesis of riboflavin (B2), biotin (B7) and folate (B9) (Fig. 14), while *M. mitochondrii* and *Rickettsia* spp. (*R. buchneri* and *R. peackokii*) are not able to synthesise riboflavin, lacking the entire pathway for this vitamin (however *M. mitochondrii* is able to synthesize the FMN and FAD cofactors, from which riboflavin acts as a precursor which might be provided by other bacteria; Sassera et al., 2011).

B vitamin-provisioning symbiont					Obligate blood feeder host	B vitamins								
Phylum	Order	Family	Strain	KEGG code		Thiamin (B_1)	Riboflavin (B_2)	Nicotinic acid (B_3)	Pantothenic acid (B_5)	Pyridoxine (B_6)	Biotin (B_7)	Folate (B_9)	Cobalamin (B_{12})	
γ-Proteobacteria	Legionellales	Coxiellaceae	*Coxiella* str. CRt	cey	Tick (*Rhipicephalus turanicus*)									
			Coxiella str. CeAS-UFV	cend	Tick (*Amblyomma sculptum*)									
			Coxiella str. CLEAA	cea	Lone star tick (*Amblyomma americanum*)									
		Legionellaceae	*Legionella polyplacis*	les	Rat louse (*Polyplax serrata*)									
	Thiotrichales	Francisellaceae	*Francisella persica*	fper	Soft tick (*Argas arboreus*)									
	Enterobacterales	Erwiniaceae	*Wigglesworthia glossinidia*	wbr	Tsetse fly (*Glossina brevipalpis*)									
			Wigglesworthia glossinidia	wgl	Tsetse fly (*Glossina morsitans*)									
		Morganellaceae	*Arsenophonus lipopteni*	asy	Deer ked (*Lipoptena fortisetosa*)									b
		Enterobacteriaceae	*Cand.* Riesia sp. GBBU	rig	Gorilla louse (*Pthirus gorillae*)									
			Cand. Riesia sp. GBBU	rip	Human body louse (*Pediculus humanus humanus*)									
α-Proteobacteria	Rickettsiales	Midichloriaceae	*Cand.* Midichloria mitochondrii	mmn	Castor bean tick (*Ixodes ricinus*)									
		Anaplasmataceae	*Wolbachia* str. wCle	les	Bed bug (*Cimex lecturalis*)									

[a]KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways used for B_1, B_2, B_3, B_5, B_6, B_7, B_9, and B_{12} are 00730, 00740, 00760, 00770, 00750, 00780, 00790, and 00860, respectively. Black squares: putatively functional pathways; gray squares: incomplete pathways with pseudogenes or missing genes; white squares: pathways absent.

[b]Only flavin reductase (*fre*) present: this enzyme converts Aquacob(III)alamin to vitamin B_{12}.

Figure 14 – **Biosynthetic pathways for B vitamins in different genomes of symbiont associated with obligate blood feeders** (from Duron and Gottlieb, 2020)

Figure 15 – **Evolutionary relationships, origin and structure of a horizontally transferred biotin operon** (from Duron and Gottlieb, 2020)

Interestingly, while it seems to be the most common shared pathway between all of the endosymbiont of obligate blood-feeders (Duron and Gottlieb, 2020), some genes are missing in the biotin pathway of *Rickettsia* endosymbiont of *I. pacificus* (REIP), being only able to synthesise the folate among the three B vitamins mentioned above (Hunter et al., 2015). However, this folate synthesis capacity of REIP seems to be disproved by the metabolic analysis of 86 *Rickettsia* genomes (including REIP, *R. buchneri* and *R. peackokii*). Indeed, the proposed hypothesis of the "FolB bypass" by the enzyme Ptps (Hunter et al., 2015) was not verified, leading to the conclusion that none of those 86 *Rickettsia* were able to synthesize correctly tetrahydrofolate (Driscoll et al., 2017). It can be added that *Lariskella*, a genus widespread in arthropods including ticks but quite rare (Duron et al., 2017; Matsuura et al., 2012), is notably associated with *I. pacificus* (Buysse and Duron, 2021; Jia et al., 2020), a species close to *I. ricinus* usually classified in the "ricinus complex" (Xu et al., 2003). It has recently been hypothesized that *Lariskella* could be the main nutritional symbiont of *I. pacificus* given its phylogenetic proximity with *Midichloria* (member of the Midichloriaceae family) (Buysse and Duron, 2021). Hence, the provision of B vitamins by Rickettsiales endosymbionts (which encompass *Rickettsia*, *Midichloria* and *Lariskella*) seems to be more limited compared to FLE and CLE.
M. mitochondrii have the ability to encode the full set of gene orthologs (bioC, FabF, FabG, FabZ, FabI, BioH, BioF, BioA, BioD, BioB) to biosynthesize de novo the full set of enzymes necessary for biotin production (B7) and almost the full set (FolC, FolK, FolP, FolC, ThyA, GlyA, FolD, fmt; folA seems lacking) for folate (B9) according to its genome (Sassera et al., 2011). Most of the genes in this biotin pathway are located closely together (except for the BioA gene) and form an operon relatively conserved across non-phylogenetically related symbionts, associated to various arthropods displaying different feeding behaviours (Fig. 15; Duron and Gottlieb, 2020; Říhová et al., 2017; Sassera et al., 2011). Furthermore, this operon seems notably widespread within *Wolbachia* mutualists (Gerth and Bleidorn, 2016; Ju et al., 2020; Nikoh et al., 2014). The mechanism of acquisition through horizontal gene transfer (HGT) of this genomic structure by *Midichloria* is not currently known. It would be tempting to speculate that the hymenopteran parasitoid *Ixodiphagus hookeri* (Chalcidoidea, Eulophidae), known to harbour *Wolbachia* in almost 100% of its individuals and frequently found within the body of *I. ricinus* (Plantard et al., 2012) could be involved in such an HGT. However, the genome of the *Wolbachia* associated with this insect has not been investigated to date. Beside this hymenopteran parasitoid, given that *Midichloria*-like DNA has been sporadically detected within the bed bug *Cimex*

lectularius (Richard et al., 2009), another hematophagous arthropod that could share a common mammal host with *I. ricinus* during its blood meal. This insect is also known to harbour the *Wolbachia w*Cle strain (i.e. *Wolbachia* endosymbiont of *C. lectularius*), an Anaplasmataceae bacteria (a sister family of Midichloriaceae, also part of the order Rickettsiales) known to possess this particular operon (Nikoh et al., 2014). Thus, this operon could have been transferred from the *w*Cle strain to *Midichloria*, in accordance with the 'intracellular arena' hypothesis (Bordenstein and Wernegreen, 2004). However, within the *C. lectularius host*, research such as the *Midichloria* prevalence, density, potential relationship or coinfection with *Wolbachia* has never been explored. It should be noted that 16S rRNA metagenomic investigations of this insect never revealed any operational taxonomic unit (OTU) associated with *Midichloria*, but rather identified the *Rickettsia* genus, along with the common association of *Wolbachia* and an unnamed Gammaproteobacteria endosymbiont of *C. lectularius* (Bellinvia et al., 2020).

Collectively, these data question which B vitamins are really necessary for tick development, which could differ between soft ticks and hard ticks (notably due to the differences between the feeding behaviours). The required vitamins may not even be the same for Metastriata compared to Prostriata, as other hematophagous arthropods seem to rely sometimes on other B vitamins provided by their respective symbionts (Kirkness et al., 2010; Michalkova et al., 2014; Moriyama et al., 2015; Nikoh et al., 2014; Pachebat et al., 2013; Rio et al., 2012; Snyder and Rio, 2015).

Despite increasing genomic evidence based on the analysis of genomic data, only FLE has been experimentally demonstrated as a B vitamin provider for the soft tick *Ornithodoros moubata*, deficiencies being rescued by oral supplementation (Duron et al., 2018). The use of an artificial blood-feeding system, like the one used in the previously mentioned paper, is relevant for testing different diets (Kröber and Guerin, 2007; Perner et al., 2016b). However, such a method appears to be more challenging for hard ticks than the conventional way of feeding on laboratory animals such as rabbits (Almazán et al., 2018). In particular, while soft ticks such as *O. moubata* feed for several short blood meals of less than one hour each, the long-lasting blood meal of hard ticks, such as *I. ricinus* (6 to 12 days), implies the use of antibiotics (and fungicide) in the blood of the artificial feeding system to prevent bacterial (and fungal) contaminations and therefore disturbs the smooth running of the experiment, hampering the development of a control treatment

in the experimental design without any antibiotics.

If not a B vitamin provider, what are the other options as a mutualistic bacteria for *Ixodes ricinus*?

Midichloria mitochondrii has the entire gene pathway to synthesize heme (Sassera et al., 2011). Heme is a prosthetic group of numerous enzymes involved in a variety of biological processes such as cellular respiration, detoxification of xenobiotics or redox homeostasis (Furuyama et al., 2007; Kořený et al., 2013) and is therefore an important cofactor. Ticks does not possess the heme pathway and cannot synthesize it unlike other Insecta and Acari (Gulia-Nuss et al., 2016; Perner et al., 2016b). Heme has been shown to be acquired during the blood meal via hemoglobin (Perner et al., 2016b) and is believed to be stored in specialized organelles called hemosomes (Lara et al., 2005; Lara et al., 2003). The vast majority of heme is thus acquired during blood meals, when for instance the female tick ingests more than 100-fold its weight of concentrated blood. I notably found that the "heme binding" MF GO term was significantly enriched in the ovary of wild-type unfed ticks carrying the endosymbiont (chapter III). The heme biosynthesis by *M. mitochondrii* in the ovary is certainly used for its own, but one could hypothesize that the symbiont could also provide heme to ovarian tick cells, at least in limited amount, notably during the off-host life periods (e.g. questing phases). Despite having a different feeding behaviour compared to ticks, such an idea has been also considered for the *Wolbachia* endosymbiont of the filarial nematode *B. malayi* (Luck et al., 2016; Wu et al., 2009). Other ticks symbionts have the genomic capacity to synthesize heme (FLE, *Rickettsia* endosymbionts; Driscoll et al., 2017; Gerhart et al., 2016) but not CLE (Moses et al., 2017). However, *C. burnetii*, a pathogen closely related to CLE (Duron et al., 2015), is considered to have acquired heme biosynthesis ability through horizontal gene transfer (Moses et al., 2017). Besides heme, *M. mitochondrii* has the genomic capabilities to synthesize other cofactors such as coenzyme A, lipoic acid or ubiquinone (Sassera et al., 2011) and could eventually provide those metabolites to its host.

The defense against natural enemies is a typical phenotype of secondary symbionts (Oliver et al., 2014). Ticks have numerous natural enemies (Samish et al., 2004). The parasitoid wasp *I. hookeri* is one of them (Hu et al., 1998) and is frequently found in *I. ricinus* (mostly nymphs; Plantard et al., 2012). It could be tempting to speculate that *M. mitochondrii* could protect female nymphs (or male nymphs harbouring sufficient amounts of

the symbiont) against *I. hookeri*. No specific negative correlation between the presence of *M. mitochondrii* and *Wolbachia* or *Arsenophonus* (which could be considered as markers of *I. hookeri* parasitism, although *Arsenophonus* is observed at a lower prevalence than *Wolbachia* in *I. hookeri*; Bohacsova et al., 2016; Plantard et al., 2012) has been found (Lejal et al., 2020a). This hypothesis implies that the emergence of males at the adult stage would be reduced, but a recent study showed that the *I. hookeri* parasitism does not apparently have a dramatic effect on tick population dynamics (Krawczyk et al., 2020). Furthermore, no major bias of *I. ricinus* sex-ratio distortion has been reported in the wild (but see for instance Cerný, 1977; Macleod, 1932; Meeüs et al., 2002), at the contrary of *I. arboricola* for instance (Van Oosten et al., 2018). On the other hand, *Spiroplasma ixodetis* which is another symbiont found at an intermediate prevalence in *I. ricinus* (Binetruy et al., 2019a; Duron et al., 2017) and density compared to other bacteria of the *I. ricinus* microbiota (Lejal et al., 2020a) could protect nymphs against *I. hookeri*, as similarly reported in *Drosophila melanogaster* (Xie et al., 2014).

Several studies have shown that bacterial pathogens were able to modulate the gut microbiota of ticks (Abraham et al., 2017; Narasimhan et al., 2014) and influence or bypass the tick immune system (Fogaça et al., 2021; Narasimhan et al., 2021). Even if we can consider that ticks harbour different specialized microbiota, stable or not, according to which organ is considered (Guizzo et al., 2020; Pollet et al., 2020), we can hypothesize that bacterial endosymbionts, especially if they are also found in salivary glands (as it is the case for *M. mitochondrii*), may interact with other bacteria, pathogenic for the vertebrate host or not. The modulation of vector capacities notably by the interaction with the main endosymbionts likely happens. As a matter of example, *M. mitochondrii*, but this time associated with *Amblyomma maculatum*, was positively correlated with the rickettsial pathogen *R. parkeri*, to the detriment of FLE. This was interpreted as a synergic association between the two bacteria, *M. mitochondrii* being essential for the growth of *R. parkeri*, and therefore improving the vector competence of *A. maculatum* for this specific bacteria (Budachetri et al., 2018). In *I. ricinus*, the same reasoning about a potential role of *M. mitochondrii* in vector competence could be applied with *R. helvetica*, both bacteria being positively associated (Lejal et al., 2020a).
All these new findings highlight the need of jointly using the traditional PCR detection method (with eventually qPCR quantification), targeting a specific genus or species of bacterium, along with metagenomic approaches allowing the investigation of the whole

microbiome at once, to grasp symbiont-arthropod interplays.

Influence of *Midichloria mitochondrii* on its host transcriptome

Despite this is not the only question that Chapter III discusses, this chapter notably tries to untangle how the *M. mitochondrii* presence or absence influences the transcriptome of *I. ricinus*, in three organs, namely the ovary, which is the main site of *M. mitochondrii* presence, but also the salivary glands (where *M. mitochondrii* has been detected in much less extent than the ovary) and the Malpighian tubules where *M. mitochondrii* has also been detected (Olivieri et al., 2019) with also a significantly reduced density compared to the ovary. The latter organ — whose physiology and role are poorly known in the case of ticks until now — is known to be one of the main site of other tick symbionts in other species, where a likely nutritional supply happens (Buysse et al., 2019; Duron et al., 2018; Lalzar et al., 2014). Thus we believed that there was a reasonable interest to investigate the *M. mitochondrii* influence in this organ, even indirectly.

This work has been complex regarding several factors. First, it is unclear how the density of *M. mitochondrii* could influence the gene expression of *I. ricinus*. As *M. mitochondrii* titers are known to increase upon engorgement (Sassera et al., 2008), it is tempting to speculate that more genes could be influenced by higher *M. mitochondrii* loads. This is not what has been observed (Chapter III), with for instance around two times more genes being overexpressed in the ovaries at the unfed stage compared to the partially-fed stage.

Among the possible biases of this study, we made a strong assumption by not considering the diversity of the microbiome but focusing on the sole *M. mitochondrii* bacteria. We had the will to perform a metagenomic study confirming the microbiome deficiency or poverty of the Lab line (Neuchâtel, Switzerland). However, amplification of bacterial 16S rRNAs using universal primers failed for the Lab line (work currently ongoing; 16S amplification of other individuals for both lineages is planned but constitute a bias). Only the absence of *M. mitochondrii* (because of qPCR amplification failure; or quasi-absence considering our results described in chapter III reporting some limited amplification in one out of the three replicates) in this lineage was confirmed. The ongoing project of the

I. ricinus genome sequencing project led notably by Claude Rispe (BIOEPAR, INRAE-Oniris, Nantes, France) uses the very same Neuchâtel (Lab) line given its high inbreeding level. Even if it is not its main purpose, whole genome sequencing is an indirect tool to determine the microbiome composition of the Neuchâtel line. A priori analyses confirmed the absence of *M. mitochondrii*, but also the microbiome poverty, with no other known tick symbiont genus replacing Midichloria (Rispe, pers. comm.). The very existence of the Neuchâtel ticks (as well as the MARB line mentioned in Chapter II) questions the obligatory nature of the symbiotic relationship between *I. ricinus* and *M. mitochondrii*. Indeed, even though we have not quantified it by properly measuring life-history traits, no apparent issues were noticed regarding the engorgement of the nymphs or adults and viability of the larvae after hatching of the Neuchâtel line (but some recent evidence of fitness cost have been observed for the MARB line; Plantard, pers. comm.). Nonetheless, further work is needed to answer this question more formally.

Another secondary issue is the genetic variability observed within the wild strain and to a lesser extent within the Neuchâtel strain (known to be highly inbred). This genetic disparity could potentially lead to biases during the de novo assembly reconstruction. Indeed, for instance, multiple contigs could have been considered as different alleles, and the read mapping might be therefore distorting the differential expression analysis. This is something that is very difficult to disentangle. One possible way to solve or at least reduce this problem is to create our own aposymbiotic strain, compared to the symbiotic strain with a very close genetic background. Therefore, attempts to create such strain have started before the beginning of my thesis. WT ticks have been collected and subjected to either several injections of marbofloxacin (MARB line), a carboxylic acid derivative third-generation fluoroquinolone antibiotic, or injections of PBS 1X, as a control. We currently have at our disposal F2 adults from both lineages. However, we have encountered some difficulties during the rearing of this tick strain (with abnormally long odd periods of moulting). RNA sequencing and metagenomic profiling could not have been performed in time in the frame of this thesis.

In parallel, as my thesis is part of a larger project coordinated by Davide Sassera, colleagues from Melbourne, Australia, are trying to answer a question related to my PhD topic, namely the characterization of both *I. ricinus* and *M. mitochondrii* transcriptome via a dual-RNAseq approach over different time points of the engorgement of *I. rici-*

nus females. However, to date, they faced different methodological issues that prevented the experiment from succeeding. Regarding those difficulties and as the *M. mitochondrii* genome is published for several years (Sassera et al., 2011), I also proposed a targeted RNAseq approach (Jones et al., 2018) to solely sequence the transcriptome of *M. mitochondrii*. I thought to eventually investigate the same three mentioned organs (mainly the ovary, but also Malpighian tubules and salivary glands) at different time points (before engorgement, and 1, 4 and 7 days after the deposit on the host) to untangle the possible tissue tropism of *M. mitochondrii* overtime of the tick life cycle, notably by the suggested localized provision of B vitamins and cofactors (Duron and Gottlieb, 2020). In that aim, ticks have been fed accordingly on vertebrate blood, organs have been dissected in January-February 2019 (the engorgement have been led in parallel to the *I. ricinus* transcriptomic approach; Chapter III) and frozen at –80°C until further use. However, due to the lack of time, the other priorities and the inherent cost of this sequencing project, this scheme has been put on hold.

Conclusion

The *M. mitochondrii* situation seems to be more complex compared to other tick symbionts such as FLE and CLE. The same remark can be aroused regarding the apparent microbiome complexity of *I. ricinus* and also of the genus *Ixodes* in general. A focus has been made specifically on *M. mitochondrii* in *I. ricinus* being the most common endosymbiont of this species (Aivelo et al., 2019; Guizzo et al., 2020; Lejal et al., 2020a; Lejal et al., 2020b). Other maternally inherited endosymbionts are found in *I. ricinus*, certainly with less prevalence and should not be neglected, neither with the rest of the species constituting the bacterial diversity. In one hand, *Rickettsia* sp. and *Spiroplasma ixodetis* (Duron et al., 2017; Lejal et al., 2020a; Sprong et al., 2009) are likely "true" maternally inherited bacteria, while *Wolbachia* and *Arsenophonus* presence are likely to be caused by *I. hookeri* parasitism (Bohacsova et al., 2016; Plantard et al., 2012). The complex interactions between all these bacterial species are still a matter of research, and each one could play a different role if mutualism is involved. However, as more and more frequently reported in other animals including arthropods, *I. ricinus* could be only partially or optionally dependent on its microbiome (Hammer et al., 2017; Hammer et al., 2019).

Midichloria mitochondrii possible mutualism has not been proven or disproven. And yet,

the unique intramitochondrial tropism occuring in *I. ricinus* and some other tick species (Beninati et al., 2004, Floriano et al., in prep), which could be interpreted as a parasitic behaviour, is not dependent on the almost fixed association characteristic within a species (Beninati et al., 2009; Gofton et al., 2015). Multiple *Midichloria* spp. closely related to the only described species could exhibit different symbiotic associations with their host (Buysse and Duron, 2018; Cafiso et al., 2016). Putting a label on *Midichloria* symbioses is not an easy task.

Appendices

Appendix A

Sequence diversity and evolution of a group of iflaviruses associated with ticks

A.1 Foreword

This study is subject to an article recently published by the journal *Archive of Virology* (ARVI).

Reference

Daveu, R., Hervet, C., Sigrist, L. et al. **Sequence diversity and evolution of a group of iflaviruses associated with ticks**. *Arch Virol* (2021). https://doi.org/10.1007/s00705-021-05060-8

This work originated from the analysis of several transcriptomic data sets produced by the lab including several ones from my PhD work.
In the frame of a project on the synganglion transcriptome of *Ixodes ricinus*(Rispe et al., in prep.), Claude Rispe performed the *de novo* assembly of this RNAseq raw data and discovered a complete genome of an Iflaviridae, a family of positive-strand RNA viruses frequently found in arthropods.
After producing my own *de novo* transcriptome assembly (see Chapter III), I checked for the presence of the same virus and found a very similar genome sequence. We thus decided to search all the already published RNAseq assembly (in the TSA database) at the seeking of related viruses and conducted phylogenetic investigations on those sequences. The results of this investigation have been formalised by the writing of this article, jointly drafted by Claude Rispe and me.

A.2 Context

With the more and more frequent discovery of full genomic virus sequences in high throughput transcriptome sequencing data, there is a growing interest in those viruses (Shi et al., 2016). Among them, Iflaviruses (from the order Picornavirales) were shown to be associated with diverse insect species at first, notably with the Deformed Wing Virus infecting honey bees (Miranda and Genersch, 2010), but have also been discovered in other arthropod subphyla (Miranda et al., 2021; Valles et al., 2017), including Chelicerata, and more specifically ticks (Kobayashi et al., 2020; O'Brien et al., 2018; Nakao et al., 2017). However, to date, the Iflaviruses associated with ticks have not shown evident symptoms related to the infection and the specific effects on host fitness (whether those viruses could be parasitic, commensalistic or mutualistic) are still unrevealed, alongside their modes of transmission. The knowledge of the distribution of these viruses within different tick species/genera and their phylogenetic relationships may help to understand the transmission mode and to describe their evolutionary history. In that aim, we screened the TSA database that comprises already published *de novo* tick transcriptome assemblies with some of our own assemblies for new genome sequences.

A.3 A possible link between Iflaviruses and *Midichloria mitochondrii*?

This chapter is not directly related to the *M. mitochondrii–I. ricinus* interaction, but I took advantage of my *de novo* RNAseq assembly to discover a complete genome of an Iflavirus.

One particular observation has not been developed in the following chapter/article. The raw reads of my data set were mapped against the viral sequence and those reads were found to be associated with the Neuchâtel (Lab) libraries for all organs, the tick strain known to be devoid of *M. mitochondrii* (Chapter III); while no reads corresponding to the viral contig were found in the Chizé (WT) libraries. Meanwhile, the transcriptomic data set based on synganglions from *I. ricinus* was obtained from wild individuals of the Chizé forest, France, the very same location as the wild ticks used for my transcriptomic study (Chapter III), which harbour *M. mitochondrii*. Therefore, among a given population, the presence of the virus seems to not be ubiquitous. Related to the presence of *M. mitochondrii*, it should be noted that the presence of related Iflavirus sequences (O'Brien et al.,

2018) have been found in *Ixodes holocyclus*, an endemic Australian tick species, which also harbours at least two different strains of *Midichloria* sp. (the sequenced fragments of the 16S rRNA slightly differ from the published genome of *Candidatus* Midichloria mitochondrii — IricVA strain) and are the dominant bacteria of the bacterial microbiome of this species (Chandra and Šlapeta, 2020; Gofton et al., 2015). For both tick species, the prevalence of Iflavirus appears to be intermediate, perhaps even rare. Although the data considered here are limited, they suggest that there is not any particular antagonism between *Midichloria* and the Iflavirus harboured by *I. ricinus* and *I. holocyclus*. The presence of one does not seem to exclude the presence of the other. The intra- or extracellular localization of Iflaviruses is unknown (while *Midichloria* are intracellular, as almost all other Rickettsiales). Thus it is difficult to speculate if any interaction can exist between the virus and the bacterium. Nonetheless, interactions with the rest of the (bacterial) microbiota are possible, even if the demonstration of such a hypothesis is delicate.

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ORIGINAL ARTICLE

Sequence diversity and evolution of a group of iflaviruses associated with ticks

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Abstract

We studied a group of tick-associated viruses with characteristics of members of the family *Iflaviridae*, a family of viruses frequently found in arthropods. Our aim was to gain insight into the evolutionary dynamics of this group of viruses, which may be linked to the biology of ticks. We explored assembled RNA-Seq data sets for different species of ticks. We identified members of five different iflavirus species, four of them novel, and discovered nine new genome sequences, including variants. Five variants represented a virus species associated with *Ixodes ricinus*. Unexpectedly, a sequence found in the *Ixodes scapularis* cell line ISE6 was nearly identical to the sequences of *I. ricinus* variants, suggesting a contamination of this cell line by *I. ricinus* material. Analysing patterns of substitutions between these variants, we detected a strong excess of synonymous mutations, suggesting evolution under strong positive selection. The phylogenies of the viruses and of their tick hosts were not congruent, suggesting recurrent host changes across tick genera during their evolution. Overall, our work constitutes a step in the understanding of the interactions between this family of viruses and ticks.

Introduction

Ticks are blood-feeding parasites, which makes of them a "hub" for a number of microorganisms (bacterial pathogens and symbionts, viruses, protozoa) that potentially interact with ticks and their vertebrate hosts. The discovery of new RNA viruses has rapidly increased in recent years, and they appear to be far more prevalent and diverse than previously expected, especially in invertebrates [1, 2]. High-throughput transcriptome sequencing (or RNA-Seq) has proven to be the tool of choice for characterizing the genomes of these viruses in many types of arthropods [2, 3], including ticks [4, 5], as this technique does not require known sequences to be targeted, while allowing the retrieval of contigs that

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may contain full viral genome sequences, given a sufficient coverage and sufficient prevalence of the virus in the analysed samples. The *Iflaviridae* are a family of RNA viruses within the order *Picornavirales.* They are non-enveloped and have a positive-sense, single-stranded non-segmented genome of ~9-11 kb [6]. This family of viruses has been shown to have a privileged association with insects, being detected in a growing number of species belonging to several orders. Iflavirus infections can range from asymptomatic to severe, causing developmental anomalies (e.g., deformed wing virus in honey bees) or death [6]. Iflaviruses have also been detected in parasitic mites (subphylum Chelicerata) [7]. Recently, genomes of viruses belonging to the family *Iflaviridae* have been detected in pools of tick species [2], in an endemic Australian tick species (Ixodes holocyclus iflavirus [IhIV]) [8], in a tick species associated with marine birds, *Ixodes uriae* [9, 10] (Gerbovitch virus), and in the ISE6 tick cell line (Ixodes scapularis iflavirus [ISIV]) [11]. Overall, members of the family *Iflaviridae* are among the most common viruses that have been identified in different species of ticks, although many aspects of their biology are still unknown, including their mode of transmission, their effect on the fitness of the tick host, and the phylogenetic relationships among members of this family. For this reason, we decided to explore the many transcriptome data sets that have been published for different tick species, searching for new sequences of members of the family *Iflaviridae*. We identified nine new genome sequences representing five different species of tick-associated iflaviruses, including several variants of the same virus species in *Ixodes ricinus*, and studied the evolution and phylogenetic relationships of this group.

Materials and methods

Search for iflavirus sequences

We searched for sequences matching iflaviruses in public tick transcriptome assemblies (available from TSA, GenBank, as of August 2020). For each transcriptome assembly, we searched for matches using tblastn [12], using the polyprotein sequence of ISIV (accession number BBD75427) as a query and RNA-Seq contigs (nucleotide sequences) as targets. Of note, several of these transcriptomes were produced by our research group for a phylogenetic study of hard ticks [13] or for expression profiling studies of *I. ricinus* (BioProject accession numbers PRJEB40724 and PRJNA662253). We used low-stringency matching criteria (e-value threshold 1-e3) to ensure that even relatively distantly related sequences could be found. Since new genome sequences identified in our study were retrieved from transcriptome data, they represent uncultivated virus genomes (UViGs). Genomes were defined as representing novel species if their sequence identity to members of existing species was less than 90%, according to the species delineation criteria of the International Committee on Taxonomy of Viruses (ICTV) for iflaviruses (https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/iflaviridae). We also performed a BLASTp search on the nr database, selecting all of the matches described as being associated with ticks and adding them to our data set. This approach ensured that our study included all of the tick-associated iflavirus genome sequences published to date (as of September 2020).

Phylogeny methods

Based on a recent phylogenetic study of iflaviruses [14], we included two outgroups, one associated with the mite *Tetranychus truncatus* (representing the closest outgroup to tick-associated iflaviruses) and the other associated with *Apis mellifera* (deformed wing virus [DWV]). We aligned amino acid sequences of all iflavirus polyprotein sequences using MUSCLE in MEGA X [15]. This alignment was filtered using Gblocks [16] to exclude poorly aligned regions and gaps. The filtered alignment comprised 1,149 amino acid positions. A phylogenetic ML tree was constructed using IQ-TREE [17]. The best model of substitution was determined using Model Finder [18], and branch support was assessed using 1000 ultrafast bootstrap replicates [19]. A graphical representation of the consensus ML tree was made using ITOL [20].

Test of congruence between host and virus phylogenies

To test the congruence between virus and host phylogenies, we used the cophylogeny testing tool Jane 4 [21]. The host phylogeny was derived from a previous transcriptome-based phylogenetic study of hard ticks, using a large number of nuclear markers [13]. Three iflavirus genomes, obtained from pools of different species of ticks, could not be included in this analysis because it was impossible to assign a host species to these iflaviruses. We also collapsed branches containing virus sequences that were nearly identical in the virus phylogeny, since they can be considered to represent the same species. This resulted not only in eliminating two iflavirus sequences associated with *I. holocyclus* but also another group of six sequences, five of which were found in *I. ricinus* and one of which was found in a cell line of *I. scapularis* (see "Results"). The name given to this group was IricIV-ISIV.

Estimation of genetic distances and evolutionary rates

We estimated genetic distances and evolutionary rates within two ensembles of sequences, one including ISIV and *I. ricinus* variants and the other including two variants associated with *I. holocyclus*, that grouped closely in the phylogenetic analysis. We first estimated genetic distances (number of substitutions per site) at both the nucleotide level and the amino acid level, using the maximum composite likelihood model [22] and the Poisson correction model [23], respectively, with the complete deletion option (all gaps excluded). This analysis was performed in MEGA X [15]. We then determined the ratios of non-synonymous to synonymous substitutions (dN/dS) using Codeml [24] with the one-ratio model for the ISIV + *I. ricinus* group and the pairwise estimate of dN/dS for the two variants found in *I. holocyclus*.

Virus presence across different populations and stages of Ixodes ricinus

The abundance of virus genomes was assessed by mapping RNA-Seq reads to the genome sequence of the iflavirus IricIV-1. Because a very low abundance of a virus can be caused by library cross-contamination (index hopping), a library was considered positive only if the read count per million was above one, following an approach used in a recent study [10].

Results

Iflavirus sequences identified

We found five variants of a novel iflavirus associated with *Ixodes ricinus* (Tables 1 and 2). These sequences were associated with different tissues or stages of the tick life cycle (in the GIDG transcriptome assembly) and with ticks from different geographical regions. All viral genome sequences obtained from *I. ricinus* were highly similar to the ISIV sequence obtained from a cell line of *I. scapularis* (~98% amino acid identity, while higher variation was detected at the nucleotide level, as shown below). Although the ISIV sequence was found in the transcriptome of a cell line from *I. scapularis*, there was no match with this sequence in the three available *de novo* assemblies obtained for three independently sequenced transcriptomes of *I. scapularis* (two of which were obtained from large collections of wild ticks, with a total of ~200 males and females from three locations for the GGIX TSA assembly). Novel iflaviruses were also identified in *Ixodes frontalis* (IfronIV), *Ixodes vespertilionis* (IvespIV), and *Hyalomma dromedarii* (HydromIV) (Table 2). For the first two of these viruses, the retrieved sequences were incomplete (5' partial), and the sequenced region of the ORF comprised 2,437, and 1,945 amino acids, representing ~81% and ~65% of the complete genome, respectively. In the case of HydromIV, the match with the ISIV sequence was in two frames, suggesting a frameshift. This frameshift could be authentic, especially if this sequence corresponds to an endogenous viral element (EVE), given that EVEs do not necessarily maintain open reading frames [25]. A detailed analysis of reads mapping to the region of the frameshift showed that it was located in a homopolymeric (poly-A) region and that the reads were polymorphic in that region, most containing a deletion of one A compared to the contig. The corrected sequence, based on the majority of reads, contained an intact ORF and no frameshift. We therefore corrected this contig, and all subsequent analysis of this sequence was based on the corrected sequence. Additionally, a 5' partial sequence was also found in a transcriptome assembly of *I. holocyclus* (2,039 amino acids, IhIV-2). This sequence was similar but not identical to that of the first iflavirus genome (IhIV) identified for that tick species. The new sequences were deposited in the GenBank database (accession numbers in Table 1) with metadata specifying the source of these viruses, the assembly methods used, and the quality of the sequence data (following the guidelines for the Minimum Information about an Uncultivated Virus Genome, or MIUVIG [26]).

All other assemblies obtained from multiple genera of hard and soft ticks gave negative results. Finally, other sequences were found after a BLASTp search against the GenBank protein database (nr), which allowed the inclusion of sequences from viruses associated with *Amblyomma americanum* (lone star tick dicistrovirus), *Haemaphysalis flava* (HfFV), *Hyalomma asiaticum* (Bole Hyalomma virus), and *Ixodes holocyclus* (IhIV), *Ixodes uriae* (Gerbovich virus) as well as viruses from pools of tick species (Table 1).

Genome organization

We analyzed the genome organization of the four sequences we consider possible representatives of novel iflavirus species (Fig. 1). Two of them (for IricIV and HydromIV) appear to contain a complete ORF and both 5' and 3' UTRs. These sequences contain conserved domains, including three capsid domains, followed by a helicase, a peptidase, and an RNA-dependant RNA polymerase (RdRp) domain. Two other sequences (from IfronIV and IvespIV) were incomplete at the 5' end, lacking the 5' UTR and at least part of the capsid domains, while the other domains and the 3' UTRs were present.

Table 1 List of iflavirus sequences found in tick transcriptomes (with tblastn or blastp, using the ISIV polyprotein sequence as a query). Columns: Species, Tissues (HEM: haematocytes, MG: midgut, MT: Malpighian tubules, OV: ovaries, SG: salivary glands, SYN: synganglion, WB: whole bodies) followed by details on the stages or conditions between parentheses, when available, Location of the sampling (or source of the strains), Accessions: GenBank accessions, protein and nucleotide, and if available, related TSA or BioProject accession between parentheses, Publication (or authors of the sequences), Percent identity with ISIV -% id at the amino acid level of the first hsp (tblastn)- and query range of the match. Lines in bold correspond to the nine iflavirus genome sequences newly discovered in the present study. For HydromIV, the sequence used was a corrected contig sequence

Species	Tissues and conditions	Location	Accession numbers	Publication	Identity	Match range(s)	Virus name
Amblyomma americanum	WB (wild questing ticks)	NY and Connecticut, USA	ASU47553.1, KX774633.1	Tokarz et al. 2018	65.8%	1142-2838	Lone star tick dicistrovirus
Haemaphysalis flava	WB	Japan	BBK20270.1, LC483655.1	Kobayashi et al. 2020	45.6%	63-2937	HflFV
Hyalomma asiaticum	WB	China	APG77501.1, KX883729.1	Shi et al. 2016	48.4%	379-2990	Bole Hyalomma asiaticum
Hyalomma dromedarii	**SG, wild ticks**	**Tunisia**	**BK012003 (GFGI01)**	**Bensaoud et al. 2018**	**40.0%**	**1-1880 and 2000-2989**	**HydromIV**
Ixodes frontalis	**WB**	**Carquefou, France**	**QPI13027.1, MT008333 (PRJNA528282)**	**Charrier et al. 2019**	**41.1%**	**560-2989**	**IfronIV**
Ixodes holocyclus	SG,WB,MG	QLD and NSW, Australia	AQZ42314.1, KY020412.1 (GIBQ01)	O'Brien et al. 2018	62.2%	1-2989	IhIV
Ixodes holocyclus	**WB**	**NSW, Australia**	**QPI13026.1, MT008332 (PRJNA528282)**	**Charrier et al. 2019**	**65.4%**	**913-2989**	**IhIV-2**
Ixodes ricinus	**SYN (wild unfed ticks)**	**Chizé, France**	**QPI13029.1, MT008330.1 (PRJEB40724)**	**Rispe et al (unpub.)**	**97.7%**	**1-2991**	**IricIV-1**
Ixodes ricinus	**SG, OV, MT (feeding adult females)**	**Neuchâtel (Switzerland), lab strain**	**QPI13030.1, MT008331 (PRJNA662253)**	**Daveu et al (unpub.)**	**98.4%**	**1-2991**	**IricIV-2**
Ixodes ricinus	**WB (various stages)**	**Czech Republic**	**BK012002 (GIDG01012278)**	**Vechtova et al. (unpub.)**	**98.1%**	**1-2991**	**IricIV-3**
Ixodes ricinus	**WB (wild nymphs)**	**Switzerland**	**QPI13031.1, MT050463 (PRJNA662080)**	**Rispe et al (unpub.)**	**98.3%**	**6-2991**	**IricIV-4**
Ixodes ricinus	**WB (wild nymphs)**	**Nancy, France**	**QPI13032.1, MT050464 (PRJNA662080)**	**Rispe et al (unpub.)**	**98.2%**	**1-2991**	**IricIV-5**
Ixodes scapularis	Cell line ISE6	-	BBD75427.1, LC094426.1	Nakao et al. 2017	100.0%	1-2991	ISIV
Ixodes uriae	WB	Antartic peninsula	QIS88066.1, MT025175.1	Wille et al. 2020	42.0%	383-2991	Gerbovich virus
Ixodes vespertilionis	**WB**	**Maine-et-Loire, France**	**QPI13028, MT008334 (PRJNA528282)**	**Charrier et al. 2019**	**68.2%**	**1016-2989**	**IvespIV**
Pool of tick species	WB	China	YP_009336552.1, NC_032764.1	Shi et al. 2016	38.6%	381-2991	Ht-V1
Pool of tick species	WB	China	YP_009336542.1, NC_032758.1	Shi et al. 2016	44.3%	391-2991	Ht-V2
Pool of tick species	WB	China	YP_009336533.1, NC_032751.1	Shi et al. 2016	46.1%	63-2989	Ht-V3

Phylogeny

The iflavirus genome sequences included in our work are described in Table 1, including nine newly identified sequences from the present study (Fig. 2). The best-fit model (BIC criteria) was LG+G4. An ML tree (consensus from 1,000 bootstrap trees) showed that the five variants found in *I. ricinu*s and the ISIV sequence grouped closely

Table 2 Percentage of amino acid identity between iflavirus polyprotein sequences, for each pair of sequences included in our study. In bold, sequences newly identified in this work. Values shaded in gray indicates identity above 90%, defining sequences that belong to the same species based on species delineation criteria of iflaviruses.

	IrivIV-1	**IricIV-2**	**IricIV-3**	**IricIV-4**	**IricIV-5**	ISIV	IhIV	**IhIV-2**	**IvespIV**	Ht-V2	Ht-V3	HyasiIV	AamerIV	Ht-V1	**HydromIV**	**IfronIV**	IuriaeIV	HflavIV	TtruIV
IrivIV-1																			
IricIV-2	99.1																		
IricIV-3	99.5	99.3																	
IricIV-4	99.4	99.6	99.6																
IricIV-5	99.6	99.2	99.7	99.5															
ISIV	99.4	99.0	99.4	99.3	99.5														
IhIV	78.3	78.2	78.3	78.2	78.3	78.2													
IhIV-2	78.3	78.2	78.3	78.2	78.3	78.2	99.8												
IvespIV	79.2	79.1	79.5	79.1	79.5	79.3	76.2	76.3											
Ht-V2	54.3	54.2	54.1	54.2	54.1	54.2	55.2	55.3	55.1										
Ht-V3	59.0	59.1	59.0	59.1	59.0	59.1	58.6	58.6	60.1	53.4									
HyasiIV	57.8	57.9	57.8	57.9	57.9	57.8	58.2	58.1	58.0	52.1	63.5								
AamerIV	57.4	57.2	57.4	57.4	57.4	57.5	56.9	56.9	59.1	52.3	58.2	55.7							
Ht-V1	47.2	47.1	47.2	47.2	47.2	47.2	48.9	48.9	48.4	46.6	46.2	47.3	45.0						
HydromIV	47.0	47.2	47.0	47.0	47.1	47.1	46.8	46.9	47.3	46.8	45.9	47.3	44.5	67.4					
IfronIV	49.2	49.3	49.2	49.2	49.3	49.3	49.3	49.3	49.0	47.2	49.1	49.6	46.1	64.0	63.7				
IuriaeIV	49.1	49.2	49.0	49.0	49.2	49.2	48.4	48.4	48.3	47.9	46.9	47.7	45.6	67.2	66.0	64.2			
HflavIV	59.6	59.7	59.5	59.7	59.6	59.7	59.1	59.1	60.2	53.2	90.0	63.7	58.6	45.9	45.4	49.3	47.2		
TtruIV	32.3	32.5	32.3	32.5	32.4	32.3	31.6	31.6	31.7	30.9	30.1	32.3	28.6	29.9	30.8	29.2	29.8	30.0	
Dwv	28.1	27.9	27.9	27.9	28.0	28.1	29.8	29.8	28.6	26.0	26.5	27.7	27.5	25.4	27.4	27.9	27.1	26.0	25.0

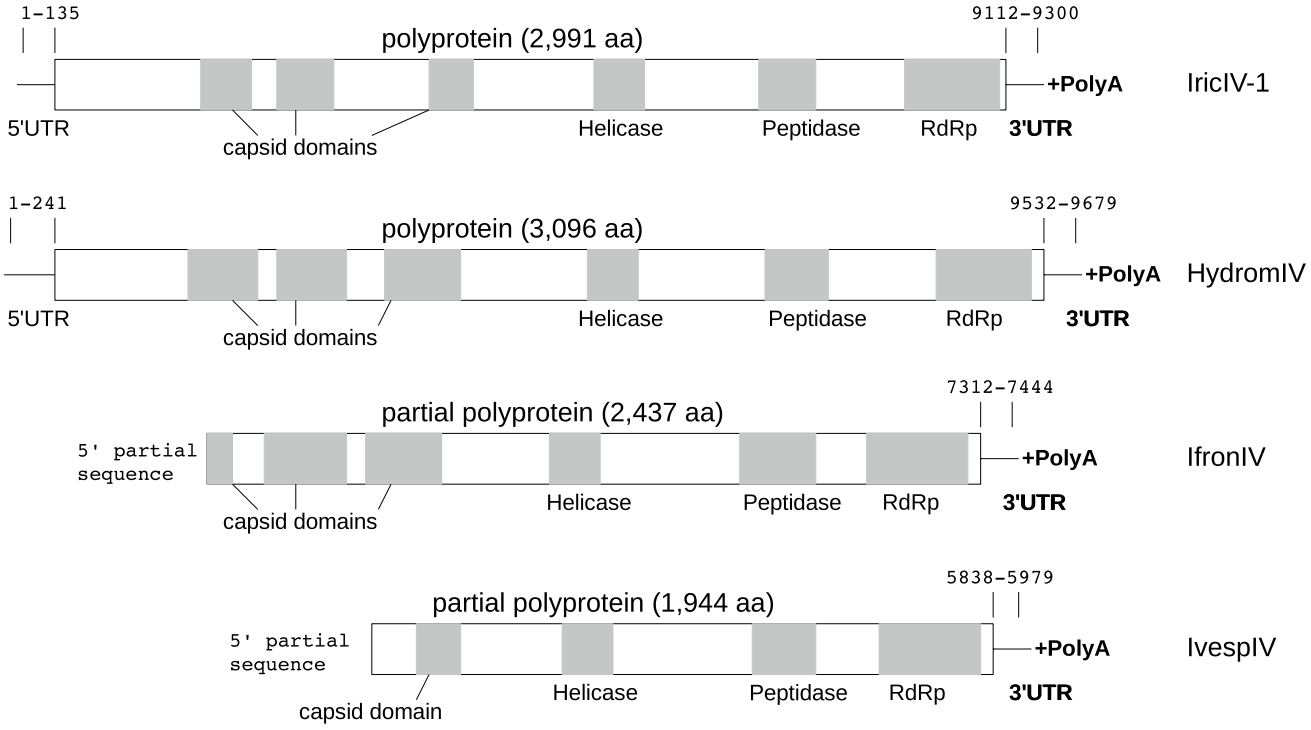

Fig. 1 Genome organization of the four novel tick-associated iflaviruses discovered in this study, respectively found in *Ixodes ricinus* (IricIV-1), *Hyalomma dromedarii* (HydromIV), *Ixodes frontalis* (IfronIV), and *Ixodes vespertilionis* (IvespIV). ORFs were predicted and domains were searched with Interproscan [34]. Predicted domains are shown as grey bars.

together (see below for details on genetic distances). This group therefore represents variants of the same virus species. This was also the case of the two variants of *I. holocyclus*. Another iflavirus sequence, found in *I. vespertilionis*, formed a sister group with the two groups above. Two other iflavirus genomes, found in *I. frontalis* and *H. dromedarii*, respectively, grouped with Ht-V1 [2]. The latter sequence was obtained from a pool of tick species that contained members

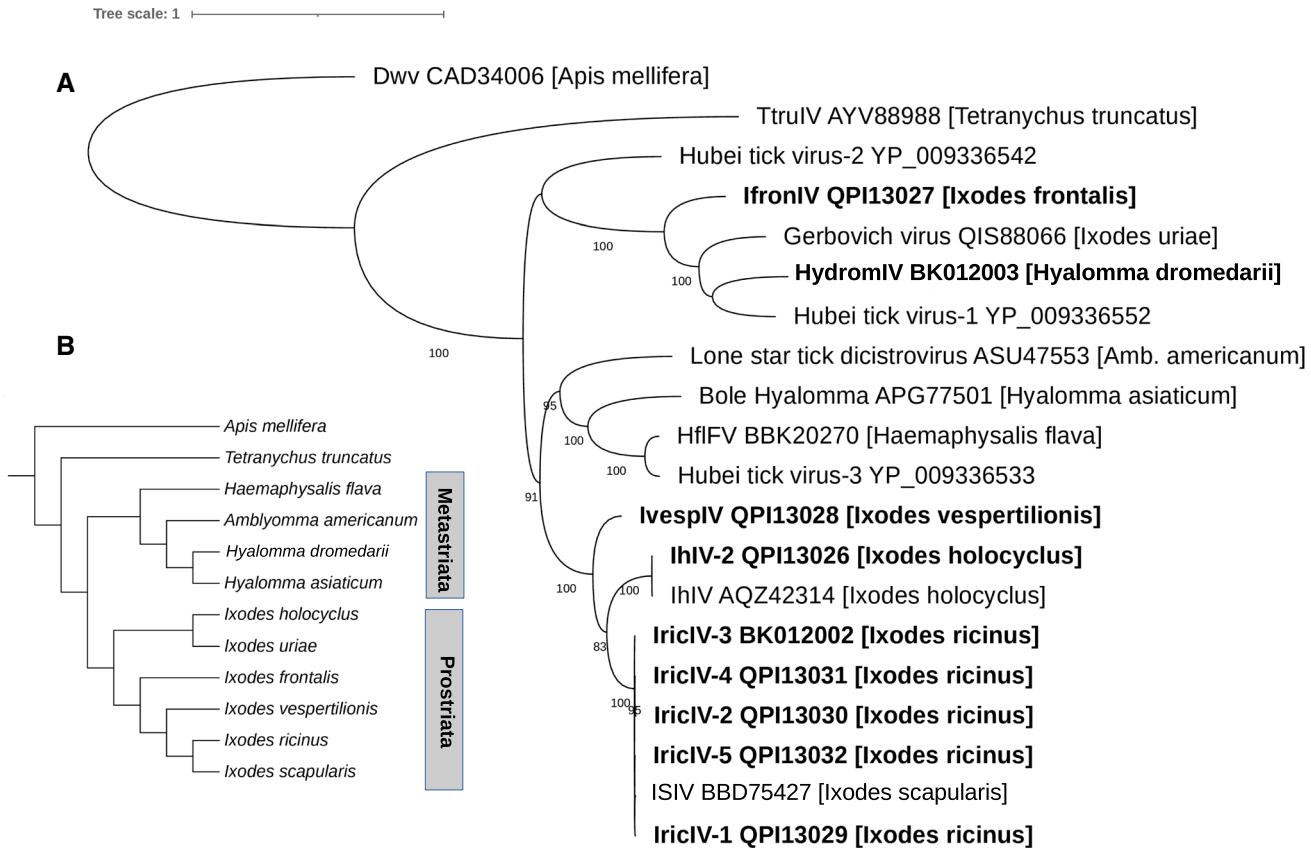

Fig. 2 **A.** Maximum-likelihood phylogenetic tree of tick-associated iflaviruses. The tree was based on the amino acid sequence of iflavirus sequences found in the transcriptomes of different hard tick species (Acari; Parasitiformes; Ixodida; Ixodidae) and rooted with two outgroups, a honey bee iflavirus (deformed wing virus [DWV]) and an iflavirus associated with *Tetranychus truncatus* (Acari; Acariformes; Prostigmata), TtruIV. The host taxon is indicated in brackets. Details related to each taxon are given in Table 1. Taxon names in bold correspond to the nine iflavirus sequences discovered in the present study. Bootstrap support is indicated at the nodes. **B.** Expected topology of the phylogenetic tree of arthropod hosts of iflaviruses included in this study, based on reference 13 and on the delimitation of the two groups recognized within the family Ixodidae (Prostriata and Metastriata).

of several species of the Metastriata (non-*Ixodes* hard ticks) or soft tick species (Argasidae), but no members of the genus *Ixodes* [2]. Of note, a sequence found in *A. americanum* in a study by Tokarz et al. [5] corresponds to an iflavirus, although it was named "dicistrovirus" in that publication.

Test of cophylogeny

A test of cophylogeny was performed using Jane 4, allowing us to depict several possible coevolutionary scenarios, all of which had the same scoring and differed very little in structure (we present one of them in Fig. 3). For all scenarios, there was an initial event of cophylogeny due to the fact that all iflavirus genomes associated with ticks form a monophyletic clade, but after this conserved ancestral node, all scenarios included four instances of host switching. An additional anomaly was a "failure to diverge" between viruses associated with *I. ricinus* and *I. scapularis* (see "Discussion").

Distances among iflavirus genome sequences and rates

For the group comprising variants found in *I. ricinus* and the ISIV sequence, the estimated pairwise distances varied between 0.010 and 0.078 at the nucleotide level, suggesting that these sequences are closely related but not identical, whereas amino acid distances were lower, ranging between 0.004 and 0.024 (Table 3). Consistently, the estimated ratio of non-synonymous to synonymous substitutions was low and well below one (with the one-ratio model, dN/dS = 0.024). The sequences of the two variants found in *I. holocyclus* were extremely similar but not identical (105 differences over 6,117 nucleotide positions, but only five differences at the amino acid level). For this pair of variants, the estimated pairwise distances were 0.018 (nucleotide level) and 0.002 (amino acid level), respectively, whereas the estimated ratio of non-synonymous to synonymous substitutions was also very low (pairwise ratio, dN/dS = 0.014).

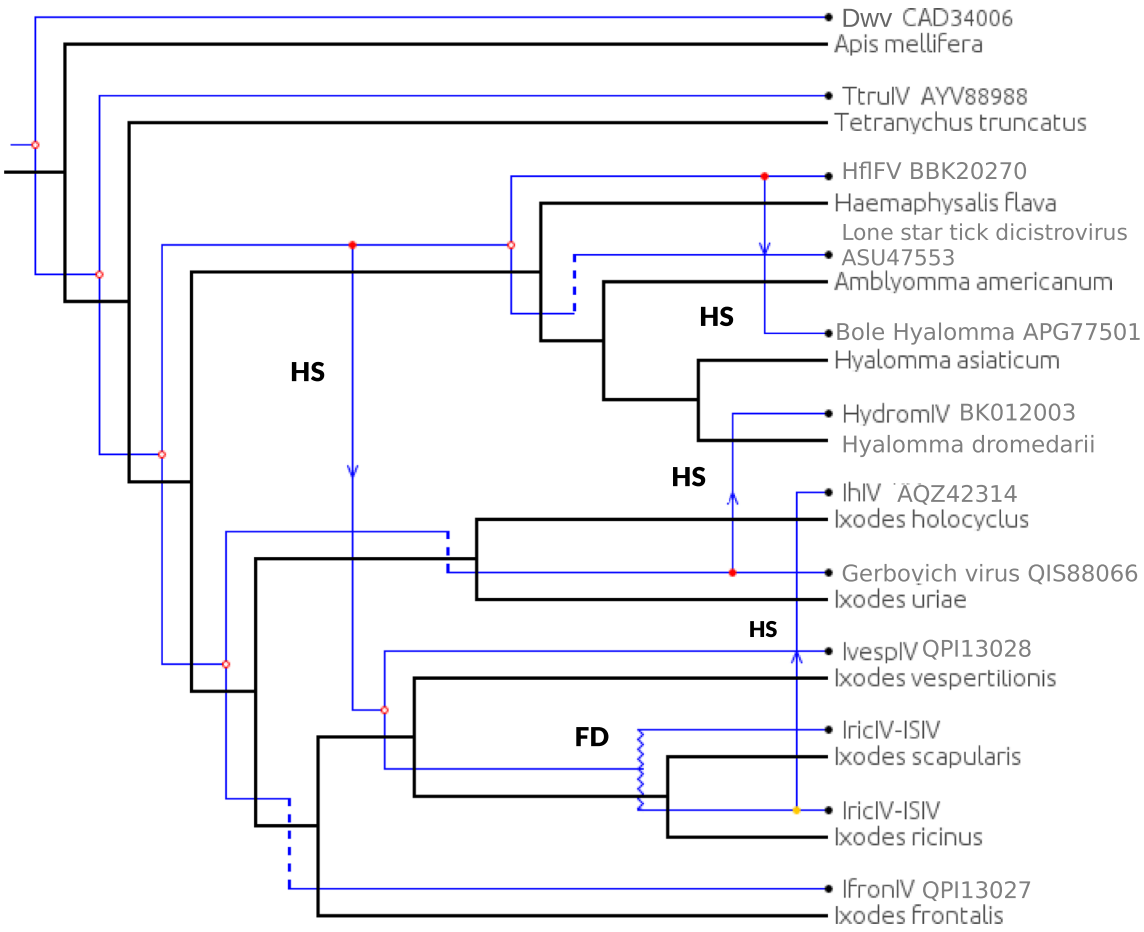

Fig. 3 Test of the congruence between the phylogenies of tick-associated iflaviruses and of tick hosts, using Jane 4. This tree contains only part of the sequences analysed in Fig. 2 (three virus genomes found in pools of tick species could not be included). Iric-ISIV represents iflavirus sequences found either in *I. ricinus* or in a cell line of *I. scapularis* (these sequences being nearly identical). Open circles at the nodes indicate cophylogeny, HS and solid colored circles, red or yellow, indicate a host shift, FD indicates a failure to diverge

Table 3 Pairwise distances among complete genomes of iflavirus associated with *Ixodes ricinus* (five variants, IricIV-1 to 5, first identified in the present study) or the *I. scapularis* cell line ISE6 (ISIV). Number of base substitutions per site (Maximum composite likelihood model, 8,976 positions) followed by number of amino acid substitutions per site (Poisson correction model, 2,991 positions). Analyses were conducted with MegaX

	IricIV-1	IricIV-2	IricIV-3	IricIV-4	IricIV-5	ISIV
IricIV-1						
IricIV-2	0.059 / 0.015					
IricIV-3	0.078 / 0.020	0.064 / 0.015				
IricIV-4	0.060 / 0.016	0.010 / 0.004	0.066 / 0.016			
IricIV-5	0.076 / 0.019	0.065 / 0.015	0.028 / 0.006	0.066 / 0.017		
ISIV	0.077 / 0.024	0.049 / 0.016	0.065 / 0.019	0.050 / 0.017	0.062 / 0.019	

Evaluation of the prevalence of iflaviruses in different populations and stages of I. ricinus

We detected the presence of the iflavirus associated with *I. ricinus* in only two out of 12 individual field populations: those from northwestern France and Switzerland. The virus abundance was null or very low in the remaining populations (i.e., below our detection threshold). In a second data set corresponding to a lab strain, the virus was detected in all life stages and conditions, ranging from a low abundance in larvae to a maximum abundance in

Table 4 Relative abundance of an iflavirus associated with *I. ricinus*, in different field populations and life-stages. Abundance was assessed by read counts per million in different RNA-Seq libraries, comparing A) different field populations (each library was obtained from a pool of 50 nymphs), data from BioProject PRJNA662080 B) different life-stages and conditions, for a lab-reared strain, data from BioProject PRJNA595586. A dot indicates a negative library (i.e. less than the one read per million threshold), where the virus is presumed absent from the pool of individuals

A)

	Germany	Spain	Finland	France-W	France-E	Hungary	Ireland	Romania	Czech Rep.	Sweden	Switzerland	UK
Read count per million	.	.	.	.	1396.54	.	.	.	.	.	553.30	.

B)

	Egg	Larvae	Fed nymphs	Females	Fed females
Read count per million	7077.10	19.39	183.65	890.34	287.20

the egg stage (Table 4), where reads assigned to the virus represented around 0.7% of all reads sequenced.

Discussion

Communities of microorganisms associated with ticks are receiving special attention in the context of increasing concern about tick-transmitted diseases [27]. In recent years, there has been a growing interest in viruses associated with ticks, and iflavirus-like viruses have emerged as some of the most common viruses in ticks [2, 5, 8, 11, 14]. Our study, based on the exploration of assembled transcriptomes, allowed us to discover nine new iflavirus genome sequences in ticks, representing five different species of tick-associated iflaviruses. We then performed a phylogenetic analysis that included these new genome sequences as well as previously published sequences. This analysis shows that the iflavirus genome sequences associated with ticks are closely grouped together and form a monophyletic clade. This suggests an ancient association between this virus subgroup and ticks, which could mean relatively infrequent host switches of the virus between major groups of arthropods. It is noteworthy, however, that two iflavirus-like genome sequences have been described recently in members of the genus *Antricola* [28], a genus of neotropical soft ticks that have a peculiar biology, being associated with hot bat caves and feeding partially on bat guano instead of having an exclusive vertebrate blood diet. These two sequences (not included in our phylogeny) grouped closely with insect iflaviruses and not with sequences associated with ticks, showing that there has been more than one infection of ticks by viruses of this family. Within ticks, the virus phylogeny and the tick phylogeny were not congruent, i.e., there was no strict co-cladogenesis (Figs. 2 and 3). One monophyletic group comprised sequences found in *I. ricinus*, *I. holocyclus*, and *I. vespertilionis*, suggesting that they form an "*Ixodes*" subclade among the iflaviruses associated with ticks. However, the virus sequence found in *I. frontalis* did not group with this ensemble and was closer to iflavirus genomes found in ticks of other genera. Also, the closer grouping of *I. holocyclus* and *I. ricinus* was inconsistent with the phylogeny of this genus [13, 29], since *I. holocyclus*, a species belonging to the Australasian subgroup of *Ixodes,* is phylogenetically distant from *I. ricinus* and *I. vespertilionis*. Although the data for iflaviruses in genera other than *Ixodes* is still relatively scarce, we finally note that the sequences associated with two species of the genus *Hyalomma* did not group together. In addition, a formal test of cophylogeny (Fig. 3) suggested several events of switching of the virus between tick species or genera over evolutionary time. A more precise picture of these dynamics will require a denser data set including more iflavirus genomes and a larger sample of tick species.

RNA viruses have relatively fast-evolving sequences, which was also suggested here by the relatively low sequence identity among genomes associated with different tick species. There was, however, a striking exception, concerning the sequence found in a cell line of *I. scapularis* (ISIV), which formed a tight cluster in the ML phylogenetic tree with five different variants identified in *I. ricinus*. In fact, these sequences were nearly identical at the amino acid level (>98% pairwise identity of protein sequences). Therefore, ISIV and the *I. ricinus* iflavirus sequences represent different strains of the same virus – a puzzling result given that the tick host species *I. scapularis* and *I. ricinus* have evolved on two different continents (eastern North America and Europe) and are separated by several million years of divergence. The geographic distance and the scarcity of animal movements (birds would be the only shared potential tick hosts) between western Europe and North America leaves little chance of a recent natural inter-species contamination through shared tick hosts. We thus propose that a contamination could explain this anomaly. Indeed, ISIV was found in the transcriptome of a cell line of *I. scapularis* (ISE6), but we failed to detect it in any other assembled transcriptomes of this species, including a data set comprising as many

as 200 adult ticks from several locations [30]. In addition, another study assessed the presence of viral sequences in *I. scapularis* after surveying several pools of wild samples (a total of > 1,100 individuals, combining nymphs and adults) from different locations – New York and Connecticut, USA – but did not report any iflavirus-like sequence [5]. Of note, using the same methods, this study did identify an iflavirus genome in *Amblyomma americanum* (although it was named "dicistrovirus", we demonstrated that this sequence groups with members of the family *Iflaviridae*). By contrast, the five *I. ricinus* variants found in the present study were obtained from several independent samples of wild ticks (or from one lab strain initially derived from wild ticks), which strongly suggests that the *I. ricinus* iflavirus variants discovered in the present study correspond to strains of iflaviruses that naturally infect this species. Based on the above, we argue that the presence of the virus in the *I. scapularis* cell line is best explained by a contamination from a virus associated in the field with *I. ricinus.* Such contamination could have occurred due to the establishment or maintenance of cell lines from the two species in a same lab, a possibility that was discussed previously for other viruses and cell lines of *I. scapularis* used in a study by Alberdi et al. [31].

Much remains to be known about the evolutionary dynamics of the association between iflaviruses and tick species. For example, the effect of iflaviruses on the fitness of their tick hosts is unknown, as there are no reports, to our knowledge, of evident symptoms in ticks infected by these viruses. A second aspect to explore is the possibility that some of the tick-associated iflaviruses could have been incorporated into the tick genome, becoming endogenous viral elements (EVEs). The initial observation of a frameshift in the contig identified for *Hyalomma dromedarii* could suggest a scenario of incorporation into the host genome, followed by pseudogenization. However, further examination of the reads led us to correct the sequence, which in fact contains an intact ORF. For HflFV, RT-PCR and PCR analysis of DNA and RNA showed that the iflavirus sequence was only amplified from RNA, and thus that it was not derived from an EVE, but this test remains to be performed for other tick-associated iflaviruses. Other related points that need to be explored are the prevalence and modes of transmission of the virus in each of the tick species studied here. In one tick species, *I. ricinus*, the RNA-Seq data set allowed us to evaluate patterns of prevalence in different life stages or different field populations. For example, the high abundance of the virus in a library obtained from a pool of eggs suggests that the virus can be transmitted vertically. More experiments, based on testing of mothers and their egg mass, will be needed to determine whether this is the exclusive mode of transmission. Another data set for *I. ricinus*, based on twelve field populations (pools of nymphs) showed that the virus was only present in a minority of populations. Based on this observation, the negative results obtained for most species (i.e., all transcriptomes of 12 species of the Metastriata and three *Ornithodoros* spp.) should not be taken as evidence of absence of this family of viruses from the entire species. Our study supports the hypothesis of an ancient association and relatively widespread presence of iflavirus genomes in tick species. We suggest that a more systematic use of RNA-Seq, based on large pools of wild individuals, would maximize the chance of detecting sequences of tick-associated viruses, even in cases of low prevalence [32]. More generally, this would enhance progress of the description of the virome associated with ticks, comprising viruses that are pathogenic for their vertebrate hosts, including humans [33].

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Declarations

Conflict of interest The authors declare no conflict of interest.

Availability of data and material Genome sequences of iflaviruses have been submitted to the GenBank database.

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Appendix B

Foreword related to the article: 'Where to find questing *Ixodes frontalis* ticks? Under bamboo bushes!'

The following study has been published in the journal *Ticks and Tick-Borne Diseases* (TTBD).

Reference

Plantard, O., Hoch, T., Daveu, R., Rispe, C., Stachurski, F., Boué, F., Poux, V., Cebe, N., Verheyden, H., René-Martellet, M., Chalvet-Monfray, K., Cafiso, A., Olivieri, E., Moutailler, S., Pollet, T., Agoulon, A., 2021. **Where to find questing *Ixodes frontalis* ticks? Under bamboo bushes!** *Ticks Tick-Borne Dis.* 12, 101625. https://doi.org/10.1016/j.ttbdis.2020.101625

B.1 Foreword

Among the tick species reported to date harbouring *Midichloria* (e.g. 8 out of 22 investigated in Epis et al., 2008, 13 out of 81 investigated in Duron et al., 2017, 7 out of 17 investigated in Cafiso et al., 2016), three different types of species can be identified regarding their *Midichloria* prevalence. In some species like *Ixodes ricinus*(in Europe), *I. aulacodi* (in Africa) and *I. holocyclus* (in Australia), almost 100% of the individuals are infected by *Midichloria*, suggesting a crucial role of this endosymbiont for its arthropod

host, with a likely mutualistic relationship and a transmission following the maternal route. Most of the remaining tick species harbouring *Midichloria* exhibited a low prevalence (e.g. 1 out 93 individuals for *I. arboricola*, 1 out of 126 individuals for *I. uriae*, 1 out of 91 individuals for *Rhipicephalus decoloratus*) (Duron et al., 2017). The presence of *Midichloria* in those species could be more anecdotal or transient, following for example a recent acquisition after a blood meal on a vertebrate with *Midichloria* in its blood, as experimentally demonstrated using wild *I. ricinus* females engorged on rabbits (Cafiso et al., 2018), therefore suggesting a horizontal transmission rather than a vertical transmission. However, *I. frontalis* seems to exhibit a different pattern compared to the two tick species described above. Indeed, an intermediate level of *Midichloria* infestation has been found (ca. 60% prevalence; investigations conducted with a reduced sample size of n = 10 adult females in Cafiso et al., 2016, n = 5 non-described individuals in Duron et al., 2017). This pattern is interesting as it questions the nature of the interaction between *Midichloria* and its arthropod host.

One way to investigate this question is to study the transmission rate of *Midichloria* from infected females to their offspring. Indeed, we could hypothesize that only some of the *I. frontalis* females infested by *Midichloria* are able to transmit this endosymbiont to 100% of their offspring. Thus, the intermediate *Midichloria* prevalence rate observed at the population level would be caused by the presence of uninfected females. The intermediate level of *Midichloria* infection in *I. frontalis* could also be due to a mixture of different subpopulations with contrasted prevalence of symbionts. For example, *Midichloria* has been identified in two *Ornithodoros sonrai* subpopulations with a rather high infection rate (68 and 94%; alongside a coinfection with *Coxiella*-like endosymbiont, which prevalence is 100% in this species) while in the other three studied subpopulations, not a single individual was found positive Duron et al., 2017. Finally, *I. frontalis* females could also transmit *Midichloria* only to a part of their offspring, leading to the production of uninfected individuals alongside infected ones.

Until recently, *I. frontalis* was considered as a rare species (see for instance Bona and Stanko, 2013 and Schorn et al., 2011 reporting the first discovery of this species in Slovakia and Germany respectively), hampering new investigations on the *Midichloria–I. frontalis* relationships due to the lack of available biological material. This tick species was mostly sampled through individuals at the engorged state via the examination of birds caught

during ringing operations (mostly on blackbirds, thrushes or robins but also on numerous other bird species, including migratory ones). The discovery, by the team where I conducted most of my thesis in Nantes, France, of an *I. frontalis* population with a high number of larvae that can be collected on the vegetation during the winter (Agoulon et al., 2019) provided new opportunities to investigate this topic. For this reason, I have been involved in a new study (where I am the third co-author) to seek for other populations of *I. frontalis* that could be used to investigate its relationships with *Midichloria* and other symbionts (Plantard et al., 2021). In this paper, we reported the discovery of 28 new populations of *I. frontalis* from France (including three from Nantes' area, where I have been involved in their discovery) and Italy. We were able to demonstrate that *I. frontalis* can easily be found below bamboo bushes (using the flagging method, consisting in passing a flannel flag over the litter on which the ticks will tie with their claws).

As an extension of this study, I have used part of the biological material collected during this fieldwork to explore *Midichloria* prevalence and vertical transmission in individual *I. frontalis* larvae. This work has first requested the set-up of a suitable DNA extraction method, due to the small DNA amount extractable from a single larva, leading to a potential lack of PCR sensitivity. Two other symbionts were also found within *I. frontalis*, namely *Rickettsia* and *Spiroplasma* (Duron et al., 2017). Therefore, the possible *Midichloria* co-infections with the other symbionts were also explored.

However, due to a lack of time (along with perturbations due to the COVID-19 pandemia), I have not been able to finalise this study during the time of my PhD. The achievement of this work that I have initiated has been partially continued by a Master student supervised by Davide Sassera and Emanuela Olivieri, and involved my other PhD supervisor (Olivier Plantard). The first results suggested that only part of the investigated *I. frontalis* frontalis larvae are harbouring *Midichloria* (ca. 13%) along with other endosymbionts (*Rickettsia*: ca. 21%; *Spiroplasma*: ca. 16%), with co-infections occurring rarely (1–5%). One surprising result was that ca. 40% of the larvae were negative for the three endosymbionts. Therefore, 16S rRNA metagenomic investigations were conducted on pools of larvae. No other bacterial genus from the 10 known maternally inherited symbiont genera was detected (Bonnet et al., 2017; Duron et al., 2017).

Of note, in the frame of a collaboration of one of my PhD supervisors (O. Plantard) and

Thomas Pollet (UMR CIRAD-INRAE ASTRE, Montpellier, France) on the microbiota associated to three *Ixodes* tick species, additional investigations have been conducted on nymphs and adults of *I. frontalis* collected during this same survey ('under bamboo bushes'). The preliminary results of this 16S metagenomic approach have given congruent results with the one described above (Lejal, Plantard, Pollet, in prep).

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Where to find questing *Ixodes frontalis* ticks? Under bamboo bushes!

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ABSTRACT

Tick-borne diseases have a complex epidemiology that depends on different ecological communities, associating several species of vertebrate hosts, vectors and pathogens. While most studies in Europe are focused on *Ixodes ricinus*, other *Ixodes* species may also be involved in the transmission or maintenance of pathogens. This is the case of *Ixodes frontalis*, a poorly known species associated with different bird species such as blackbirds, thrushes and robins, with a wide distribution covering most European countries.

In a previous study, high densities of questing *I. frontalis* larvae were found during autumn-winter at a site close to Nantes (western France) where a long-term survey focused on *I. ricinus* was conducted. These *I. frontalis* were mostly observed under bamboo bushes. In the present study, we investigated the presence of *I. frontalis* under bamboo bushes at various locations. With that aim in mind, a systematic search for questing *I. frontalis* was undertaken by the flagging method in public urban parks and private gardens presenting bamboo bushes (32 sites). This survey was carried out during autumn-winter to maximize the probability of finding the most abundant stage, *i.e.* larvae. We searched for *I. frontalis* first in the area of Nantes (10 sites), then in other regions of France (21 sites) and at one site in northern Italy. A single visit to each site revealed the presence of *I. frontalis* at 29 out of 32 sites: larvae were always present, nymphs were frequent (59 % of the positive sites), while adults were found at only 14 % of the sites. Questing stages of this understudied species are thus easy to find, by dragging or flagging under bamboo bushes in autumn or winter. We make the assumption that bamboo offers a favourable place for birds to roost overnight outside their breeding period (*i.e.* spring), sheltered from both predators and wind. This would explain higher densities of *I. frontalis* under bamboo, relative to other biotopes.

As *I. frontalis* is known to harbour zoonotic pathogens, the consequences of this discovery on the epidemiology of tick-borne diseases are discussed.

1. Introduction

While most studies of tick-borne diseases including field sampling usually focus on widespread tick species known to have a strong impact on animal and public health, it is crucial to keep in mind that the eco-epidemiology of those diseases is also related to the occurrence of other tick species, even if they are considered to be rare. The most common tick species in Europe, *Ixodes ricinus*, is a generalist tick with a

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prominent role in the transmission of pathogens (responsible for Lyme borreliosis or tick-borne encephalitis as major examples) to humans. Moreover, *I. ricinus*, by its broad host spectrum, can behave as a bridge vector species able to transmit pathogens, notably to humans, that would otherwise stay confined to a more restricted association between a particular host and a more specialised tick species that would not bite humans (Heylen et al., 2017). For example, particular strains of *Anaplasma phagocytophilum* have been reported to be associated with *Ixodes ventalloi*, a nidicolous specialist tick parasitizing rabbits (Jaarsma et al., 2019), or to *Ixodes trianguliceps*, a nidicolous specialist tick infesting small rodents (Bown et al., 2006), while those hosts are also used by *I. ricinus* for its blood meals. Another example involves birds, notably blackbirds and other species of the *Turdus* genus, that are frequently parasitized by the tick *Ixodes frontalis*, an exclusively ornithophilic species (Drehmann et al., 2019; Martyn, 1988; Remesar et al., 2019a, b). As birds have often been quoted as potential reservoirs of *Borrelia* that can be transmitted to humans by *I. ricinus* bites (Coipan and Sprong, 2016; Hanincová et al., 2003), the implication of specialist ticks associated with birds, such as *I. frontalis*, in the maintenance of zoonotic pathogens within bird populations can be suspected. Besides human health, some bird diseases have also been reported as associated with *I. frontalis*, including the Chizé Virus (Chastel et al., 1999) and avian tick-related syndrome (TRS) (Monks et al., 2006).

Ixodes frontalis, sometimes called the passerine tick (Hillyard, 1996), has been detected on birds in most European countries (Estrada-Peña et al., 2017), even if reports of this species on birds are still less frequent than those of *I. ricinus* (Norte et al., 2020b). Surprisingly, questing *I. frontalis* have been rarely reported on vegetation, despite a recent increase in the number of reports (Agoulon et al., 2019; Bona and Stanko, 2013; Gillingham et al., 2020; Kahl et al., 2019; Schorn et al., 2011). The finding of free stages of *I. frontalis* remains infrequent and no description of a particular habitat, that could help to find this tick species elsewhere than on its host, has yet been provided, except by Agoulon et al. (2019). These authors discovered thousands of *I. frontalis* larvae under bamboo bushes, collected by the dragging method in autumn and winter, even revealing a higher frequency of the larval stage of this species relative to *I. ricinus* during those seasons.

In this article, to investigate if this finding can be generalised, tick samplings were carried out under bamboo bushes at several locations in France and Italy to search for *I. frontalis* questing stages.

2. Material and methods

We targeted sites presenting bamboo bushes with a minimum height of 4 m and a minimum surface of 5 m^2. The search for new sites was performed first around the initial site in western France, in the vicinity of Nantes (Agoulon et al., 2019), then at a larger scale in France, thus covering different climatic conditions, and in the vicinity of Pavia in northern Italy. Thirty-two sites were found (Fig. 1), either in public urban parks or in private gardens. A typical example of a collection site is illustrated in Fig. 2. Table 1 summarizes study site characteristics (site number and name, GPS coordinates, surface of prospected bamboo patches, sampling date, time spent on flagging) and tick counts (by species and stages).

Tick collections were performed from the beginning of November to the end of April, according to previous results from Agoulon et al. (2019), who showed that this period was the most favourable to collect questing larvae of *I. frontalis*. This stage was particularly targeted because it was the most abundant one, maximizing the probability of finding the tick species. Tick samplings were carried out during two periods: late 2017-early 2018 and late 2018-early 2019.

The flagging method was adopted, because it was more appropriate

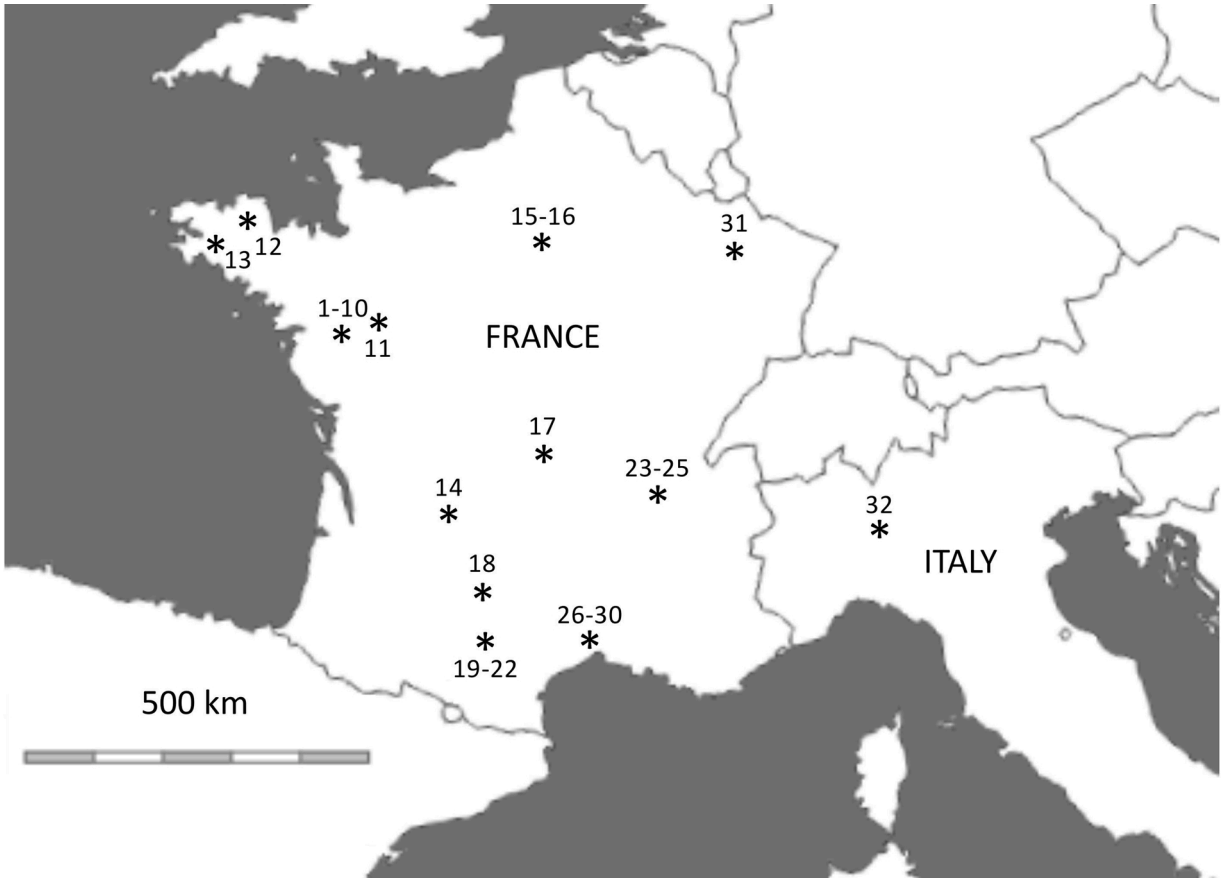

Fig. 1. Map of the 32 collection sites (*). Some sites correspond to several locations (see Table 1: sites no 1-10, 15-16, 19-22, 23-25, 26-30).

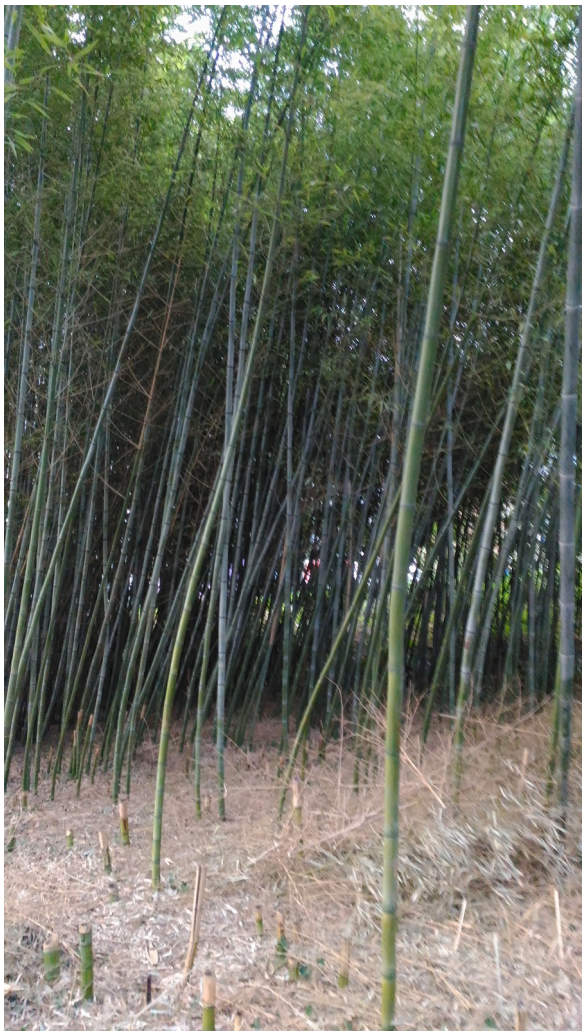

Fig. 2. Illustration of a collection site (site no 9).

for tick sampling within dense vegetation areas (see Fig. 2) than the dragging method, which requires areas free of obstacles such as trees, bushes or bamboo stems. Sampling was carried out on the ground, between bamboo stems or along trails surrounded by bamboo bushes, with a flannel flag (dimensions around 40 × 40 cm) mounted on a handle with a 45° angle. The time spent on flagging ranged from 5−160 min, including time needed to extract ticks from the flag with tweezers to put them in 70 % ethanol. Ticks were identified under an optical microscope (100X magnification) according to the morphological keys provided in Heylen et al. (2014a), Pérez-Eid (2007) and Estrada-Peña et al. (2017). A site was considered positive as soon as a single individual from any stage of *I. frontalis* was found.

3. Results

During the first period (late 2017-early 2018), we found eight locations with bamboo patches conforming to the required criteria of surface area and height in the region of Nantes, France, and all sites revealed the presence of *I. frontalis* (Table 1). During the second period (late 2018-early 2019), we extended investigations to 23 additional sites in France (2 locations near Nantes and 21 sites elsewhere in France) and one site in Italy (Table 1). All the sites, except three, were again found positive for the presence of *I. frontalis*.

A total of 2676 larvae, 247 nymphs and 7 adults (all females) of *I. frontalis* were collected during this sampling. In addition, *I. ricinus* was also occasionally found (2 larvae, 43 nymphs and 7 adults [4 females and 3 males]). Although only a limited time was spent sampling at most sites (usually less than one hour), a high number of *I. frontalis* was found, mostly at the larval stage (91 % of collected individuals were larvae). Among the 29 positive sites, larvae were always present, nymphs were frequent (59 % of the sites) and adults were only occasionally found (14 % of the sites).

4. Discussion

To the best of our knowledge, our study is the first one that clearly identifies a suitable biotope, *i.e.* below bamboo bushes, in which *I. frontalis* can be found with a very high likelihood (90 % of the 32 prospected sites). *Ixodes frontalis* ticks were not detected at only three sites. Two of them (no 14 and no 20) were probably too small (5 and 12 m^2 respectively, corresponding to the two smallest bamboo bushes of our sampling) to detect the passerine tick, perhaps because small bamboo patches may attract too few birds. At the third site (no 15), located in central Paris, the area of bamboo can be considered as atypical in comparison to the other sampled sites as it was not surrounded by any other kind of vegetation and a woven mat (geotextile) was covering the humus layer below the bushes. Thus, our results show that *I. frontalis* is widespread, at least in western Europe, and it can probably be found in most bamboo patches of a sufficient area and height.

Because *I. frontalis* is ornithophilic (Martyn, 1988), we hypothesize that bamboo bushes constitute high quality roost sites for birds, especially during the night and outside the breeding period (*i.e.* spring) of the birds used by this tick species as hosts. In particular, bamboos (Poaceae) are grasses and not trees and thus do not bear robust lateral branches that could allow terrestrial predators (including mustelids, or cats in more urban areas) to climb in the foliage and thus reach birds during their sleep. Moreover, the density of small lateral side branches in bamboo is so high that it also probably hampers predation by raptors (including both nocturnal and diurnal birds of prey such as goshawks or buzzards, and even some species that can be found in urban areas such as sparrowhawks or owls). Finally, bamboo also has persistent foliage and this characteristic allows roosting birds to save energy due to heat loss during colder and windier winter times. Because of the scarcity of biological data available for *I. frontalis,* the peak activity season of *I. frontalis* females is still debated (Agoulon et al., 2019; Doby, 1998; Heylen et al., 2017, 2014a, b). However, some observations on the passerine ticks collected on birds suggest that it may be in late summer-early autumn (Monks et al., 2006). Bamboo bushes could thus concentrate bird roosting in this period and increase the likelihood that engorged *I. frontalis* females fall to the ground beneath the bamboo, hence producing larvae in this particular habitat in autumn-winter. The bird species using bamboo as roosting sites remain to be determined, but passerines such as blackbirds, thrushes, robins, house sparrows, finches or tits are good candidates. These species are also known to be used for their blood meals by *I. frontalis* (Martyn et al., 1988). In the USA, the use of bamboo as “blackbird lure roost habitat” has been proposed to avoid local urban and agricultural blackbird problems, illustrating the attractivity of these plant species for bird roosting (Flynt and Glahn, 1993; Glahn et al., 1991). We do not mean that this tick species is exclusively associated with this particular plant (as it is also found in other places where no bamboo is present; AA, OP, personal observation), but we emphasize that it can be more easily found underneath bamboo, due to the concentration effect of this plant on birds, probably in relation to its quality as a nocturnal perching site.

Our sampling thus confirmed and generalised the results of the finding described in the Agoulon et al. (2019) paper, revealing a dramatic concentration of *I. frontalis* larvae underneath bamboo. Compared to the site of western France studied in the first paper, a higher proportion of nymphs was found at most of the different sites investigated in the present study. At their site, Agoulon et al. (2019) found a total of 1728 larvae, 28 nymphs and 5 adults (all males) of *I. frontalis* with the dragging method along trails under bamboo bushes. The generally

Table 1
Collection data of questing ticks across the 32 study sites (L = larva, N = nymph, F = female, M = male, for each tick species).

Site number	Site name (department - city - locality)	GPS coordinates	Bamboo patch surface (m^2)	Sampling date	Sampling duration (min)	*Ixodes frontalis*				*Ixodes ricinus*			
						L	N	F	M	L	N	F	M
1	44 - Nantes - Parc de la Chantrerie	47°17′18″N, 1°31′31″W	960	06/11/2017	160	552	2	–	–	–	–	–	–
				09/02/2018	30	128	5	–	–	–	–	–	–
				19/03/2018	30	104	2	–	–	–	–	–	–
				10/12/2018	60	346	1	–	–	–	–	–	–
				20/03/2019	60	262	73	1	–	–	–	–	–
2	44 - Nantes - Oniris Chantrerie	47°17′23″N, 1°31′29″W	20	01/12/2017	10	1	–	–	–	–	–	–	–
3	44 - Sucé sur Erdre - La Hérinière	47°22′29″N, 1°32′02″W	20	08/11/2017	10	32	–	–	–	–	–	–	–
4	44 - Nantes - Parc du Grand Blottereau	47°13′46″N, 1°30′24″W	540	06/12/2017	45	202	2	–	–	–	–	–	–
5	44 - Nantes - Parc de la Godinière	47°14′38″N, 1°34′47″W	900	16/12/2017	30	56	3	–	–	–	1	–	–
6	44 - Nantes - Parc de la Beaujoire	47°15′40″N, 1°31′55″W	80	09/02/2018	45	69	–	–	–	–	–	–	–
7	44 - Nantes - Jardin des plantes	47°13′08″N, 1°32′37″W	135	21/02/2018	45	50	1	–	–	–	–	–	–
8	44 - Bouguenais - La Trocardière	47°10′54″N, 1°34′27″W	610	21/02/2018	30	97	2	–	–	–	–	–	–
9	44 - Saint Herblain - Bagatelle	47°14′37″N, 1°36′59″W	800	28/02/2019	35	64	14	1	–	–	–	–	–
10	44 - Orvault - Provotière	47°15′19″N, 1°36′44″W	195	10/04/2019	50	8	16	–	–	–	2	–	–
11	49 - Angers - Terra Botanica	47°30′00″N, 0°34′10″W	475	14/03/2019	40	70	8	–	–	–	–	–	1
12	22 - Treglamus - Rumin	48°34′01″N, 3°15′12″W	50	22/04/2019	20	5	1	–	–	1	9	–	–
13	29 - Quimper - Men Foues	48°03′18″N, 4°06′03″W	100	27/02/2019	60	20	23	2	–	–	17	2	2
				28/02/2019	30	46	18	1	–	1	6	–	–
14	24 - Saint Paul de Serre - La Bouyerie	45°04′31″N, 0°37′54″E	12	16/03/2019	5	–	–	–	–	–	2	–	–
15	75 - Paris - Paris Expo Porte de Versailles	48°49′51″N, 2°17′19″E	500	03/03/2019	20	–	–	–	–	–	–	–	–
16	94 - Vincennes - Parc Floral & Tropical	48°50′15″N, 2°26′37″E	970	15/04/2019	60	9	56	2	–	–	1	–	–
17	63 - Biollet - Bambouseraie	46°00′04′'N, 2°42′41′'E	2180	22/02/2019	70	142	6	–	–	–	1	–	–
18	82 - Caussade - Rue de Lavaur	44°09′16″N, 1°32′32″E	30	29/12/2018	8	7	–	–	–	–	–	–	–
19	31 - Vieillevigne - Roumingou	43°23′49″N, 1°39′17″E	24	22/02/2019	5	2	–	–	–	–	–	–	–
				24/02/2019	5	3	–	–	–	–	–	–	–
20	31 - Vieillevigne - Chemin du Cammas	43°24′03″N, 1°39′22″E	5	23/02/2019	5	–	–	–	–	–	–	–	–
21	31 - Saint Léon - Bois de la Plano	43°25′20″N, 1°33′19″E	20	23/02/2019	15	37	–	–	–	–	–	–	–
22	31 - Montesquieu - Barrelis	43°25′47″N, 1°36′45″E	16	06/03/2019	15	3	–	–	–	–	–	–	–
23	69 - Tour de Salvagny - Rue de Sutin	45°47′52″N, 4°43′10″E	10	05/03/2019	30	2	–	–	–	–	2	1	–
24	69 - Fleurieux sur l'Arbresle - Servy	45°50′32″N, 4°39′22″E	500	05/03/2019	15	112	3	–	–	–	1	1	–
25	01 - Misérieux - Centre horticole	45°59′14″N, 4°47′45″E	1000	05/03/2019	90	12	2	–	–	–	–	–	–
26	34 - Montpellier - Vailhauquès	43°39′50″N, 3°42′38″E	130	13/02/2019	60	49	–	–	–	–	1	–	–
27	34 - Montpellier - Prades-le-Lez	43°41′29″N, 3°52′39″E	150	27/02/2019	60	12	3	–	–	–	–	–	–
28	34 - Montpellier - Plaine de Roques	43°40′52″N, 3°51′29″E	430	04/03/2019	75	3	–	–	–	–	–	–	–
29	34 - Montpellier - Lavallette	43°38′51″N, 3°52′29″E	900	05/03/2019	20	1	1	–	–	–	–	–	–
30	34 - Montpellier - Gigean	43°29′57″N, 3°42′30″E	50	05/03/2019	45	1	–	–	–	–	–	–	–
31			4370		90	152	5	–	–	–	–	–	–

(*continued on next page*)

Table 1 (*continued*)

Site number	Site name (department - city - locality)	GPS coordinates	Bamboo patch surface (m^2)	Sampling date	Sampling duration (min)	*Ixodes frontalis*				*Ixodes ricinus*			
						L	N	F	M	L	N	F	M
	54 - Nancy - Jardin Botanique	48°39′33″N, 6°09′20″E		27/02/2019									
32	27 - Pavia (Italy) - Parco Sora	45°11′40″N, 9°06′15″E	600	18/03/2019	60	17	–	–	–	–	–	–	–
Total						**2676**	**247**	**7**	**0**	**2**	**43**	**4**	**3**

higher frequency of nymphs in the present study, and also to a lesser extent of adults at certain sites, may at least be partially related to the use of the flagging instead of the dragging method: dragging is already known to let adult ticks fall more easily from the blanket when it is dragged over a certain distance (Dantas-Torres et al., 2013; Milne, 1943).

Although ticks are strictly hematophagous and do not rely on any other feeding resource than the one provided by their vertebrate host, the analysis of vegetation type has often been investigated to explain tick presence/absence or density. In particular, vegetation type acts on the habitat's microclimate and soil, which may have an effect on tick abundance and questing activity (Dobson et al., 2011; Gilot et al., 1979, 1975; Lindström and Jaenson, 2003). However, we are aware of very few studies that have reported a direct link between a particular plant species and the presence/absence or density of a tick species. This is the case for example for the non-native tree species *Solanum mauritianum* and *Ixodes holocyclus* (Buettner et al., 2013). The authors reported an outbreak of tick paralysis in an Australian population of bats (*Pteropus conspicillatus*) and hypothesized that this outbreak was linked to the introduction of this invasive new bush species that led those frugivorous bats to forage in a lower vegetation height than previously, exposing them to the bite of the indigenous tick *I. holocyclus*. The association of *I. frontalis* with bamboo could correspond to a similar relationship between a particular plant species and a particular tick species, induced by a concentration of the hosts in this particular vegetation type, favouring a higher density of this tick species. There are several reports of *I. frontalis* in urban areas, such as parks and gardens (Drehmann et al., 2019; Schorn et al., 2011). Because *I. frontalis* is found with a high likelihood in any bamboo bush of a sufficient surface area and because such bamboo bushes are especially frequent in urban areas, this tick species is probably a very common member of the tick community in such habitats). As *I. ricinus* can act as a possible bridge vector, investigations on *I. frontalis* and harboured pathogens such as *Borrelia* spp. may be relevant for public health issues (Buczek et al., 2020; Norte et al., 2020a, b). In the context of the increasing interest of the influence of urbanisation on infectious diseases (Hassell et al., 2017), including ticks and tick-borne diseases (Grochowska et al., 2020; Hansford et al., 2017; Rizzoli et al., 2014), our findings highlight the potential role of *I. frontalis* in the eco-epidemiology of tick and tick-borne diseases in such areas (Hamer et al., 2012).

Our results, favouring the sampling of this species, pave the way to additional investigations aiming at elucidating unknown aspects of the biology of this tick species, such as its population dynamics and its relationships with bird behaviour.

CRediT authorship contribution statement

Olivier Plantard: Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Thierry Hoch:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Romain Daveu:** Investigation, Resources, Writing - review & editing. **Claude Rispe:** Investigation, Resources, Writing - review & editing. **Frédéric Stachurski:** Investigation, Resources, Writing - review & editing. **Franck Boué:** Investigation, Resources, Writing - review & editing. **Valérie Poux:** Investigation, Resources, Writing - review & editing. **Nicolas Cebe:** Investigation, Resources, Writing - review & editing. **Hélène Verheyden:** Investigation, Resources, Writing - review & editing. **Magalie René-Martellet:** Investigation, Resources, Writing - review & editing. **Karine Chalvet-Monfray:** Investigation, Writing - review & editing, Funding acquisition. **Alessandra Cafiso:** Investigation, Resources, Writing - review & editing. **Emanuela Olivieri:** Investigation, Resources, Writing - review & editing. **Sara Moutailler:** Investigation, Resources, Writing - review & editing. **Thomas Pollet:** Investigation, Resources, Writing - review & editing. **Albert Agoulon:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

None.

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DOCTORAT ECOLOGIE
BRETAGNE GEOSCIENCES
LOIRE AGRONOMIE ALIMENTATION

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Santé et alimentation au cœur de la vie

Titre : Interactions entre la bactérie endosymbiotique *Candidatus* Midichloria mitochondrii et son hôte arthropode, la tique *Ixodes ricinus*

Mot clés : tiques, symbiose, mutualisme, transcriptomique, antibiotiques

Résumé : *Ixodes ricinus* est la tique la plus répandue en Europe occidentale et le principal vecteur de Borrelia burgdorferi s.l. responsable de la maladie de Lyme. Outre les pathogènes, les tiques, à l'instar des nombreux animaux, hébergent de nombreux micro-organismes non-pathogènes, dont l'effet sur leur hôte s'étend le long d'un continuum allant du mutualisme au parasitisme. Au sein de cette communauté microbienne associée à *I. ricinus*, l'Alphaproteobactérie (Rickettsiales) *Midichloria mitochondrii* est la plus répandue au sein des différentes populations de tiques. Cet endosymbiote, capable de résider à l'intérieur des mitochondries des cellules de tiques, est particulièrement abondant dans les ovaires des femelles adultes où la bactérie est systématiquement transmise à la descendance et est suspectée d'être un symbiote mutualiste obligatoire. Cependant, l'existence même des lignées de tiques dépourvues du symbiote remet en cause le caractère obligatoire de cette symbiose. Ainsi, l'étude de l'interaction *M. mitochondrii*–*I. ricinus* est d'intérêt majeur afin d'établir si la bactérie est essentielle à la valeur adaptative de la tique, ce qui constituerait un potentiel levier d'action de lutte anti-vectorielle se basant sur une lutte antisymbiotique. Dans un premier temps, la dynamique de *M. mitochondrii* entre les nymphes mâles et femelles d'*I. ricinus* a été étudiée. Dans un second temps, un traitement antibiotique (tétracycline) visant à supprimer la bactérie de son hôte ainsi que l'hypothèse d'un apport de vitamines B par *M. mitochondrii* à la tique ont été testés sans permettre d'établir de conclusions. Dans un troisième temps, une étude du transcriptome de tiques hébergeant ou non *M. mitochondrii* a permis d'identifier des gènes différentiellement exprimés pouvant être impliqués dans l'interaction tique–bactérie. La nature de la relation *M. mitochondrii*–*I. ricinus* apparaît donc encore plus complexe que celle observée chez d'autres couples tiques–endosymbiotes.

Title: Interplays between the bacterial endosymbiont *Candidatus* Midichloria mitochondrii and its arthropod host, the European tick *Ixodes ricinus*

Keywords: ticks, symbiosis, mutualism, transcriptomics, antibiotics

Abstract: *Ixodes ricinus* is the most common tick in Western Europe and the main vector of Borrelia burgdorferi s.l., the agent of Lyme disease. In addition to pathogens, ticks, like many animals, harbour numerous non-pathogenic microorganisms, whose effect on their host extends along a continuum from mutualism to parasitism. Within this microbial community associated with *I. ricinus*, the Alphaproteobacterium (Rickettsiales) *Midichloria mitochondrii* is the most widespread in the different tick populations. This endosymbiont, capable of residing within the mitochondria of tick cells, is particularly abundant in the ovaries of adult females where the bacterium is systematically transmitted to the offspring and is suspected to be an obligate mutualistic symbiont. However, the very existence of tick lines without the symbiont calls into question the obligatory nature of this symbiosis. Thus, the study of the *M. mitochondrii*-*I. ricinus* interaction is of major interest in order to establish whether the bacterium is essential to tick fitness, which would constitute a potential lever for vector control based on antisymbiotic control. Firstly, the dynamics of *M. mitochondrii* between male and female nymphs of *I. ricinus* were studied. In a second step, an tetracycline-based antibiotic treatment aimed at removing the bacterium from its host as well as the hypothesis of a B vitamins provision by *M. mitochondrii* to the tick were tested without making it possible to draw any conclusions. Thirdly, the analysis of the transcriptome of ticks harbouring or not *M. mitochondrii* made it possible to identify differentially expressed genes that could be involved in the tick-bacteria interaction. The nature of the *M. mitochondrii*–*I. ricinus* relationship thus appears even more complex than that observed in other tick–endosymbiont pairs.