

Influence of the microbiota on a phytophagous insect : interactions between Delia radicum and its intracellular and extracellular symbionts

Valérie Lopez

▶ **To cite this version:**

Valérie Lopez. Influence of the microbiota on a phytophagous insect : interactions between Delia radicum and its intracellular and extracellular symbionts. Agricultural sciences. Université de Rennes, 2018. English. NNT : 2018REN1B044 . tel-02081438

HAL Id: tel-02081438
https://theses.hal.science/tel-02081438

Submitted on 27 Mar 2019

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UNIVERSITE ECOLOGIE
BRETAGNE GEOSCIENCES
LOIRE AGRONOMIE ALIMENTATION

UNIVERSITÉ DE RENNES 1

THESE DE DOCTORAT DE

L'UNIVERSITE DE RENNES 1
COMUE UNIVERSITE BRETAGNE LOIRE

ECOLE DOCTORALE N° 600
Ecole doctorale Ecologie, Géosciences, Agronomie et Alimentation
Spécialité : « *Écologie et Évolution* »

Par
Valérie LOPEZ

Impact du microbiote chez un insecte phytophage :
Interactions entre *Delia radicum* et ses symbiotes intra et extracellulaires

Thèse présentée et soutenue à Rennes, le 30 novembre 2018
Unité de recherche : Institut de Génétique, Environnement et Protection des Plantes (IGEPP)

Rapporteurs avant soutenance :

Laurence Mouton	Maître de conférences – Université Claude Bernard Lyon 1
Christine Braquart-Varnier	Professeure – Université de Poitiers

Composition du Jury :

Rapporteures :	Laurence Mouton	Maître de conférences – Université Claude Bernard Lyon 1
	Christine Braquart-Varnier	Professeure – Université de Poitiers
Examinateurs :	David Giron	Directeur de Recherche CNRS – Université de Tours
	Hervé Merçot	Directeur de Recherche CNRS – Université Pierre et Marie Curie
Directeur de thèse :	Denis Poinsot	Maître de conférences – Université de Rennes 1
Co-directrice de thèse :	Anne Marie Cortesero	Professeure – Université de Rennes 1

Insiders Rule

***W**itchcraft was not born from your race:*
***O**urs it has been from untold times*
***L**ike goblins we have roamed the Earth*
***B**uried deeper than meet your eyes*

***A**s stowaways we pull the strings*
***C**ulling the males before they're born*
***H**ow we do it? No magic rings*
***I**t's in the deal when we are borne*

***A**t our beginnings we were free*
***P**roteobacteria of the wild*
***I**nto hosts since has grown our tree*
***P**assengers terrible or mild*

***I**n any cell we can be found*
***E**gg or soma, winter or spring*
***N**ot bearing us means shaky ground*
***T**hose who try may not bear offspring*

***I**n ovo hear this, worm or fly:*
***S**ave us — you live; shed us — you die.*

– Denis POINSOT –

– Remerciements –

Quand on commence une thèse, il est difficile d'imaginer cet instant ; celui où on aura tout juste terminé d'écrire son manuscrit de thèse ; celui où on s'assiéra devant son écran, avec le curseur qui clignote enfin sous la partie « Remerciements ». Je pensais que cet exercice serait facile, mais il ne l'est pas. J'ai peur d'oublier quelqu'un évidemment, mais aussi peur que mes mots ne parviennent pas à refléter le fond de ma pensée. Si ma crainte n°1 se réalise, honte à moi, et par avance, mes plus plates excuses pour l'oubli ! Pour remédier à la crainte n°2, je vais laisser courir mes doigts sur le clavier sans trop réfléchir, sans trop relire, et laisser parler ma spontanéité.

Je tiens tout d'abord à remercier sincèrement **Laurence Mouton** et **Christine Braquart-Varnier** pour avoir accepté d'être rapporteures de ma thèse ; ainsi que **David Giron** et **Hervé Merçot** pour leur présence en tant que membres du jury le jour de ma soutenance.

*(Pour l'anecdote, **Hervé Merçot** était le directeur de thèse de mon directeur de thèse, voilà donc une jolie façon de boucler la boucle !).*

Je remercie également chaleureusement tous les membres de mes comités de thèse pour leur bienveillance et leurs conseils précieux. Vous savez tous que mon premier comité a été assez difficile pour moi après cette première année quasiment tombée à l'eau à cause de cette satanée *Wolbachia* qui avait décidé de disparaître de l'une nos lignées de mouches. Vous avez su ne pas dramatiser la chose et me faire rebondir, et pour cela, un grand merci à **Yannick Outreman, Christophe Mougel, Nicole van Dam, Fabrice Vavre, David Renaud, Hervé Colinet, Jean-Christophe Simon et Stéphane Derocles**. Merci également à **Anne Atlan**, ma tutrice, tu as su trouver les mots justes, même si difficiles à entendre, quand j'en avais vraiment besoin.

Vient ensuite le tour de mes encadrants, mes chers encadrants ! Il va être difficile de résumer en quelques lignes toutes les jolies choses que j'aimerais dire à votre sujet, car très franchement, je n'aurais pas pu espérer avoir de meilleurs « chefs » que vous, comme je me plais à vous appeler ; je vais quand même essayer.

Denis, alias papa poule n°2. Je pense que peu de doctorant(e)s ont eu la chance d'avoir un directeur de thèse comme toi. Tu es quelqu'un de passionné et de passionnant, quelqu'un d'à part, je pense que tu vis un peu sur ta propre planète, ce qui fait de toi une personne

absolument unique ; je me sens vraiment privilégiée de te connaître. Tout a commencé lorsque j'ai repris mes études en 2011 après… hum… 5 années d'arrêt en tout. Cela a été une vraie galère administrative pour moi, et déjà à l'époque, tu m'avais sauvé la vie : c'est grâce à toi que j'ai enfin pu me réinscrire sereinement en Master 1. Professeur exceptionnel, tu t'es tout de suite démarqué ! Je t'ai dit que tu faisais peur aux étudiants, pas vrai ? Mais je t'ai aussi dit que moi, je n'avais pas peur ! Haha ! Et c'est en toute connaissance de cause que je t'ai contacté en 2015, depuis ma tente au Mexique, après avoir pédalé pendant deux ans, parce que ma nouvelle lubie, c'était de faire une thèse avec toi en rentrant ! Ni une ni deux, tu m'as concocté un sujet aux petits oignons, et bim, quelques semaines après, j'étais parmi vous. Merci donc de m'avoir donné cette chance ! Merci pour ta présence tout au long de cette thèse, pour m'avoir remonté le moral quand j'avais des coups de mou, pour avoir été là simplement TOUT LE TEMPS, et même être venu les jours fériés pendant ma rédaction, juste « au cas où j'aurais besoin de toi ». Merci pour les pains au chocolat et les chocolats chauds rapportés de la cafét' parce que « j'avais besoin de sucre » en cette fin de rédaction, merci pour ton œuvre d'art trouvée un matin sur mon bureau quand ça n'allait pas fort, je vais la garder précieusement, car ça restera un chouette pense-bête, même après la thèse ;)



Anne Marie, alias maman poule n°2. Probablement la directrice de labo la plus cool qui soit ! Scientifique accomplie, professeure, directrice de labo que tu gères d'une main de maître, tout en restant bienveillante à tous les égards. Souriante (tout le temps) et débordante de gentillesse. Ce qui m'a le plus marqué depuis le début, c'est ton incroyable disponibilité. Il y a des jours où tu étais clairement débordée, à courir à droite à gauche, avec à peine le temps de grignoter un truc le midi, et pourtant, ta porte était toujours entrouverte, et à chaque fois que j'y glissais la tête pour savoir si tu avais 5 minutes, c'est toujours un « oui », et avec le sourire bien sûr ! Merci pour ta patience, tes encouragements et ton encadrement en règle générale, pour tes remarques hyper pertinentes (qui font que certains articles ont fait pas mal d'A/R entre ma rédaction et tes corrections → cf. Article n°2, mais on a réussi !!!). Le travail c'est une chose, mais il n'y a pas que ça ! Le surnom de maman poule n°2 tu l'as bien mérité toi aussi, comme par exemple pour toutes ces fois où tu as pris des nouvelles de moi chaque jour lorsque j'étais clouée au lit. Merci pour ton sens de l'humour exacerbé, parce qu'il faut le dire, on s'est quand même bien marrées, et

j'avoue que la toute première fois que je t'ai rencontrée, j'ai senti que le courant allait passer tout de suite. Merci pour tous les verres partagés (ah cette passion commune du champagne… on ne se refait pas hein !), merci de m'avoir fait suffisamment confiance pour faire des petits trous à ta fille (bientôt ton tour, je tiens à le rappeler ! peut-être que tu pourras aussi m'aider à immobiliser Denis pour lui mettre un anneau dans le nez de force ?? hinhin), et merci également pour ta fidélité à l'Épicerie-Valou.com !

Quoi qu'il arrive, **Denis**, **Anne Marie**, j'espère très sincèrement que notre histoire ne s'arrête pas là, et que nous aurons de nombreuses occasions de nous recroiser.

Yannick, un ÉNORME merci pour ton aide et ta bienveillance. Pour moi, tu représentes vraiment "la force tranquille" à l'état pur, tu dégages cette espèce d'aura d'intelligence et tout ce que tu dis tout le temps est incroyablement pertinent, j'ai une grande admiration pour toi. J'espère avoir mon nom très bientôt à côté du tien sur une publication, puisque cette opportunité a été quelque peu retardée à cause de ma tête qui était loin ce jour-là… tu sais, sur la Lune, loin là-bas !... Merci pour ton aide très poussée sur l'analyse de mes données, et tes encouragements pendant ces 3 ans, c'est quelque chose qui m'a vraiment aidé et que j'ai vraiment apprécié, d'autant plus que tu n'étais pas du tout obligé de le faire, alors encore merci.

Maxime, alias la force tranquille n°2 ! Merci pour ton aide précieuse sur toute la partie statistique de ce manuscrit. Très franchement, je n'aurais jamais été capable de faire des scripts aussi poussés toute seule. Tu m'as fait gagner un temps précieux bon nombre de fois, et au moins avec toi je suis sûre d'avoir fait des analyses de qua-li-té ! Merci pour ta patience, ta pédagogie, et pour avoir toujours pris le temps de m'expliquer toutes ces équations/scripts qui me semblaient barbares au premier abord. Tu m'as convaincue qu'elles n'étaient pas si méchantes que ça finalement ! Merci pour ta bonne humeur et ton sourire constants, et d'avoir toujours été réceptif à mes blagues toutes pourries. Je suis bien contente que tu ne sois pas devenu maçon, on ne se serait probablement jamais croisés sinon !

Chrystelle, merci pour ton aide avec nos chères mouches ! Tu m'as sauvé à maintes reprises lorsque j'ai décidé, en 3ème année, de finalement me faire aider et d'arrêter de tout faire toute seule tout le temps ! Comme j'ai regretté de ne pas avoir crié « à l'aide » avant tant le travail avec toi a été agréable. Les mesures de pattes, les comptages d'œufs, les élevages, la biologie moléculaire... je me souviens de plusieurs journées passées au Rheu où je ne sais pas bien comment j'aurais fait sans toi ! Merci pour ton sourire radieux en toutes circonstances, tu illumines notre labo, ne change rien !

Valérie, merci de m'avoir formée à l'élevage de mouches (je ne pensais pas que je dirais un truc comme ça un jour !) et de m'avoir remplacée au pied levé de nombreuses fois auprès de mes "bébés". Merci pour les nombreuses discussions (rarement sérieuses il faut l'avouer !) et pour ton rire facile que j'entends souvent raisonner dans le labo, et qui me fait immanquablement sourire en retour.

Merci également à **Christophe** et **José** d'avoir pris le relais avec mes « bébés » à chaque fois que j'en ai eu besoin (c'est quand même inquiétant que je persiste à qualifier mes mouches de la sorte non ?). Vous m'avez sauvé la mise bien des fois !

Morgane, ç'a été un plaisir de travailler avec toi sur notre manip commune. Je te remercie par avance pour les futures analyses statistiques et bio-informatiques auxquelles tu vas être confrontée à cause de moi… niark niark ! A charge de revanche ! (est-ce que si je paye ma tournée, ça compte ?). Je suis ravie de t'avoir initiée au wakeboard même si au fond, je pense que tu m'en as secrètement voulu pour la semaine de courbatures qui a suivi. Merci de m'avoir accompagnée dans certaines autres de mes activités loufoques comme la Pole Dance, on a quand même bien rigolé à glisser comme des larves sur cette barre en fer (la sexy attitude, ça n'est pas pour tout de suite ! haha). Et après réflexion, OK, peut-être qu'on laissera Romain venir avec nous la prochaine fois, surtout s'il a promis de venir en brassière, c'est un deal !

Christophe, je pense que tu m'as fait le cadeau le plus onéreux de toute ma vie : le séquençage d'une plaque 384 puits ! Comme ça, bim ! Je pense qu'une bière fait piètre figure en comparaison, mais je te l'offrirai quand même volontiers (je vais finir par passer pour une sacrée pochtronne si je propose d'offrir des verres à tout le monde dans ces remerciements…). Merci pour cette opportunité en tout cas, et pour ta confiance dans ce projet ; je pense sincèrement que l'on va réussir à sortir un joli petit article avec tout ça.

Merci **Xavier** de m'avoir permis de recycler toutes tes pupes ! J'en ai fait bon usage promis, et en plus, tu m'as évité des dizaines d'heures d'échantillonnages sur le terrain. Tu ne sais probablement pas à quel point je déteste ça, mais crois-moi, tu m'as rendu un grand service !

Merci à **Frédérique Mahéo** et **Stéphanie Morlière** de m'avoir formée à cette discipline obscure qu'est la biologie moléculaire ; ç'a été un véritable plaisir de travailler avec vous !

Merci à tous les doctorants/étudiants du bureau 418, ça n'aurait pas été pareil sans vous. On a quand même été nombreux à y cohabiter, et pourtant, il y a régné une bonne ambiance en permanence, que l'on y travaille pour de vrai ou bien que l'on fasse semblant !

Gaëtan, ta bonne humeur nous manque dans le bureau ! Ainsi que ton accent qui me rappelait un peu la maison. Et puis, il nous manque maintenant un joueur pour éclater nos ballons à coups de scalpel-scotché-sur-un-stylo ! Je regrette également ton beau calendrier mural spécial « légumes »… Le mien avec pin-ups et beaux gosses tatoués commence à me lasser…

Fabrice, le grand dresseur de homards ! Tu es certainement la personne avec l'animal de compagnie le plus original que je connaisse. En tout cas, j'en sais un peu plus sur ces bestioles-là grâce à toi ! Merci. Aucun savoir n'est inutile franchement, imagine qu'une question sur le homard soit la grande question finale si je suis invitée un jour sur le plateau de « Qui Veut Gagner des Millions » ?! Merci pour les nombreux moments de rigolade dans le bureau, et pour les nombreuses vidéos très funs partagées (pas sûre que je puisse vraiment les citer ici, hum…). Je tiens aussi à préciser que tu en as eu de la chance d'avoir du boulot à revendre au Rheu pendant ma rédaction, tu auras ainsi évité d'avoir à subir mon stress et par conséquent, mon humeur massacrante, héhé…

Merci **Kathleen** pour nos discussions très intéressantes ! De m'avoir hébergée quand j'en avais besoin alors que tu étais en plein déménagement, et aussi pour le trèfle à 4 feuilles que tu m'as offert ; tu me croiras ou non, mais il est toujours sur mon bureau, impeccablement conservé, où il m'aura vraiment accompagné jusqu'à la toute fin !



Merci également à **Stéphane, Michaël** et **Jérôme** pour votre gentillesse et votre bonne humeur, même si vos passages par chez nous ont été assez rapides finalement.

Merci à mes stagiaires **David, Solène, Béatrice et Alexiane**, vous avez été au top ! Autonomes et appliqués, j'avoue qu'avoir commencé l'expérience de l'encadrement avec vous a été simple comme bonjour !

Merci à tout le laboratoire pour les centaines de repas pris ensemble, les cafés, les pots (car il y a toujours un truc à fêter au 4ème étage du bâtiment 25 !). J'ai rarement travaillé

dans une ambiance aussi saine et détendue, et c'est grâce à vous tous : V**éro, Carole, Marie-Françoise, Françoise, Alain, Antoine, Dennis, David et Sébastien** (ainsi que tous ceux déjà cités plus haut, évidemment !).

Thank you **Nicole** for your enthusiasm and your incredible kindness. Working with you in Leipzig was such a breath of fresh air, and honestly, I needed that very much. Everything was perfect there! I loved Germany, your lab, and the work that was so interesting. Thank you again for this great opportunity and experience. And thank you for being so much more than a supervisor when I was there, for taking me to the Tattoo & Piercing exhibition (where I could teach you things for once!) and for the great evening at your place, I will always remember it.

Thank you all at the iDiv department for welcoming me the way you did and being so incredibly nice.

Susann, you will definitely keep a special place in my heart, I'm waiting for you in Rennes next year, hurry up! And don't forget to bring you roller quads okay? Roller Disco!!!

Alexander, I had so much fun with you and so many good memories: at the greenhouse, the lunches, the Laser Tag, and of course, Roller Disco^{bis}!!! Un abrazo!

Nicola, thank you for taking care of me the way you did! For helping me with all my experiments when you had so much work to do for yourself already. Thank you for all the good times… Roller Disco!!!^{ter}

Esraa, I'm so happy I've met you, I'm also keeping the best memories of the time spent with you. Again, so sorry I made you have rum by mistake!!! I hope Allah understands that it was all my fault! ^^ I am glad you liked the crepes anyway, that was a holy sin I promise!

Rebekka, you're probably one of the nicest girl I've ever met! Thank you for everything, your help, and your smile. It's too bad we could only share a couple of days because of your holidays that were just at the same time. But I'm sure we'll see each other again, in Leipzig or in Rennes.

Esther, thank you for your help and the interesting conversations we had. I look forward to another bowl of fried ice cream with you girl!

Ellen, **Andreas, Axel, Ainhoa, Christian, Martin, and Crispus,** thank you for all the good times during lunches and of course, at the 3.00 p.m. coffees! I look forward to challenging you all again at Laser Tag!

Merci aux sites pirates **Sci Hub** et **BookSC** qui m'ont bien souvent été indispensables pour obtenir des articles introuvables autrement, et aussi parce que je crois sincèrement que la connaissance devrait être accessible à tous. Merci également au site **Ciel Mon Doctorat !** pour avoir réussi à égayer certains des aspects les plus sombres d'une thèse !

Merci à **Mélanie**, pour ton soutien sans faille ! Tu sais exactement ce que j'ai vécu tout au long de ces 3 années, puisque nous avons traversé les mêmes épreuves (avec deux ans de décalage !). Merci d'avoir cru en moi plus que moi-même ! Je dirais même « aveuglément » (ah, l'amour et ses œillères ^^). Je suis moi aussi très fière d'être ta derby wife !



Merci enfin à tous mes amis qui ont été absolument nécessaires à ma santé mentale pendant ces trois ans !

Les Bretons tout d'abord.

Merci **Marco** pour ta disponibilité, pour être toujours partant quoi que l'on fasse, et pour m'avoir sauvé la vie à mon retour de Leipzig : répondre présent sans hésiter et te taper 1h30 de route dans la foulée pour me ramener chez moi, à 21h, alors que tu étais posé tranquille en pyjou chez toi, c'était franchement bro' ! Merci pour ta bonne humeur, ton franc-parler et tes conneries à la pelle – je ne m'en lasse pas – ; je m'estime très chanceuse de t'avoir comme ami.

Elodie et Charlie, qui aurait cru qu'une amitié si forte se créerait entre nous quand nous nous sommes rencontrés au détour de nos voyages respectifs, dans un bivouac tout pourri dans les Redwoods, en Californie ? Il faut croire qu'une soirée dans le froid, sous la pluie, avec un minuscule panneau publicitaire comme abri de fortune, ça rapproche… Merci d'être venus vivre en Bretagne (après que l'on vous ait harcelé un peu, c'est vrai) et pour les nombreux week-ends passés tous les 4 qui m'ont bien permis de décompresser pendant ces 3 ans, le temps passe toujours beaucoup trop vite avec vous. Team OshiPit, je vous aime ! Mais je crois que vous commencez à être au courant vu le nombre de fois où je vous l'ai déjà dit ^^

Merci **Fanny** pour ton rire facile et ta motivation à toute épreuve, et pour t'être mise au derby aussi, pour qu'on puisse se taper un peu, mais pas trop finalement ! Merci de m'avoir fait confiance pour tous tes piercings (ça commence à faire quelques fois que je te troue ^^) et pour tous tes encouragements ! Je te souhaite bon courage pour ta « nouvelle vie » et tes nouveaux challenges, que tu réussiras de nouveau avec brio, je n'en doute pas une seconde !

Merci également à **Mathieu, Julien (David Brownie), Jeremy, Simon** ("j'aime les Kinder Pingui", tu vois, c'était easy), **Laurent, Julien (Juju), Diego, Mireia, Andres, Thib, Audrey, Eloïse, Clémence et Christopher**, pour les nombreuses sorties et bons moments passés ensemble, il serait beaucoup trop long de tous les mentionner ici !

Merci à vous tous également pour votre incroyable cadeau d'anniversaire d'il y a deux ans ! J'avais promis à mes directeurs de ne pas prendre le risque de mourir avant la fin de ma thèse, donc je n'en n'ai pas profité avant, MAIS je vais ENFIN pouvoir m'inscrire à la session d'avril prochain… À moi le Brevet A de parachutisme !!! Merci à vous !

Merci également à mes amis sudistes, **Moumoune, Topos, Ju, Piou, Julien (Ty') et Teffie.** Cela fait + de 15 ans que l'on se connait tous (Teffie remporte quand même la palme avec 25 ans !). Vous êtes tellement importants pour moi, et vous me manquez terriblement au quotidien. J'ai convaincu Moumoune de venir s'installer en Bretagne ça y est, c'est une première victoire, mais d'autres suivront, je suis tenace !

D'ailleurs, un merci particulier à **Moumoune** pour avoir corrigé une partie des remerciements de cette thèse et mon résumé en français (autant que ça serve d'avoir un copain auteur ! hé hé). *D'ailleurs, petit coupure pub : « n'hésitez pas à acheter les incroyables romans fantasy/fantastiques de Môsieur Adrien Tomas », ce sont des tueries ! Tous. Oui, tous ! Mais ne dites pas trop fort qu'il s'appelle Moumoune en vrai… il râle après… un peu…* **Moune**, j'ai souvent été la bêta-lectrice de tes romans avant publication ; sache que tu as été le tout premier à pouvoir parcourir ce manuscrit en entier ; je dirais que la boucle est bouclée.

Merci également à **Laetitia** et **Thomas** d'avoir rejoint nos rangs ! Je pense sincèrement que mes ami(e)s ont trouvé des perles rares, vous êtes incroyables ! (et ta glace au chocolat aussi Laëti ! ^^).

Et parce que le sport a fait partie intégrante de ma vie pendant ces 3 ans, merci à tous les copains **grimpeurs** de la salle Block Out à Rennes, à toute mon **équipe de Roller Derby**

(et plus particulièrement à **No Hugs, Poulpy, Wulverine, Total Ripley, Notre-Dame-sans-Pitié et Brummbär, Rage Dedans** et **Régine Boule**), et aux **"riders"** des wakeparks Plessé et La Rincerie, pour tous les week-ends sportifs, mais aussi de détente passés ensemble ! Bon là, depuis les trois derniers mois de rédaction, j'ai RIEN fait, et je suis toute grasse maintenant, va falloir remédier à ça !

Merci **Maman, Papa** et à ma soeur « **Kiki »** (hé hé, je ne suis même pas désolée !) pour avoir toujours cru en moi, en toutes circonstances. Merci pour votre soutien sans faille, c'est grâce à vous que je n'ai pas baissé les bras, je ne voulais surtout pas vous décevoir. Nous nous sommes malheureusement très peu vus ces trois dernières années (sans compter les deux d'avant où nous étions à vélo !). Vous me manquez énormément (et mon adorable neveu **Alex** aussi !). Mais promis, maintenant que nous allons de nouveau partir en vadrouille, nous allons pouvoir nous rattraper. Je vous aime.

Merci également au reste de ma famille, les taties, les tontons, belles-mamans et beau-papa ! Je suis désolée d'avoir donné si peu de nouvelles pendant ces 3 ans, mais cela ne veut pas dire que je n'ai pas pensé à vous, loin de là !

Merci à **Nyx**, alias **PitMix**, alias **Pitu**, pour m'avoir remonté le moral bien des fois, grâce à son amour absolument inconditionnel, sa mignonnerie et sa maladresse évidemment (car c'est un rare spécimen de chien avec 4 pattes gauches ! ^^).

Enfin, merci **Alexys**, le seul, l'unique, le Vinou, de 2You… Merci de m'avoir supportée tout au long de cette thèse, et bien plus encore, bientôt 10 ans (tu as vu comme j'aime bien remuer le couteau dans la plaie ? ☺). Merci d'égayer ma vie au quotidien avec toutes tes lubies, passagères ou non, tes projets loufoques, que je prends un malin plaisir à suivre, et évidemment tous les incroyables petits plats que tu me prépares chaque soir ! Merci de m'avoir accompagnée tout au long de cette thèse, car je sais que rester 3 ans sédentaire, cela t'a vraiment coûté moralement. Nous n'avions jamais fait ça avant, et c'était un pari risqué, mais on a réussi, TU as réussi, merci d'avoir tenu le coup, et pour cet énorme sacrifice que tu as fait. « C'est de l'amour !!! ». Et assez ironiquement (car je sais que tu ne peux plus la voir en peinture), cette thèse est pour toi.

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General context and main objectives of the thesis

The term "superorganisms" was coined in 1928 by the great myrmecologist William Morton Wheeler to describe a colony of eusocial insects, such as ants. Later, the "superorganism" appellation has been applied by some to symbioses between dissimilar species. Indeed, there was a need for a term defining this "living together" of different organisms without necessarily having to classify these interactions as commensalism (neutral), mutualism (beneficial), or parasitism (harmful). To better reflect this reality, Lynn Margulis proposed in 1993 that any physical association between individuals of different species for significant portions of their life history constitutes a "symbiosis", that all participants are "bionts" and that the resulting assemblage is a "holobiont".
Recently, exciting advances in biology allowed us to understand how the microbiota – the community of bacteria, fungi, and other single-celled microorganisms harbored by a host – can deeply influence its functions. It is now widely recognized that the microbiota can have a huge impact on its host physiology and behavior. Its composition and function are indeed critical for most animals and plants, so much so that many scientists believe that from now on, hosts and their microbiota should be considered as a single ecological unit: the holobiont (Zilber-Rosenberg *et al.*, 2008; Gilbert *et al.*, 2012).

According to this concept, a comprehensive understanding of the biology of a species requires that it be studied in an ecological context with its microorganisms as an important component of the system (Steinhaus, 1960). In a nutshell, it is now even argued by some that the "holobiont" (host plus its microbiota) and its constituent "hologenome" (the totality of genomes in the holobiont) are a unit of selection, and therefore this unit has properties similar to an individual organism, i.e., it is a superorganism (Zilber-Rosenberg *et al.*, 2008).

Apart from their fundamental importance to understand the evolution of species, the relationships between insects and their symbionts have also been extensively studied by many entomologists to develop new strategies based on their interactions for controlling pests. They showed for example, that the bacteria associated to the diamondback moth, *Plutella xylostella*, a major pest of *Brassica* plants and other cruciferous vegetables worldwide, were involved in increased host consumption index, relative growth rate, and even insecticide resistance (Lin *et al.*, 2015). Likewise, Zabalou *et al.* (2004) suggested

that *Wolbachia*, an intracellular symbiotic bacterium transmitted vertically by females to their offspring, could be used as an environmentally friendly tool for the control of pest populations thanks to its ability to induce a reproductive barrier called cytoplasmic incompatibility (CI) when introduced in novel hosts. CI kills the embryos of uninfected females when they mate with infected males, while infected females are compatible with both infected and uninfected males (Werren *et al.*, 2008). Thanks to CI, this team of researchers managed to completely suppress laboratory cage populations by single releases of *Wolbachia*-infected males. These results showed that introduction of this bacteria into pest and vector species could have some potential in suppressing or modifying natural populations of economic and hygienic relevance.

In the present work, we have studied the holobiont constituted by the cabbage root fly (*Delia radicum*) and its community of microbes. This fly is a major pest of brassicaceous crops (rape, cabbage, turnip...) where its belowground larvae feed on roots (Doane & Chapman, 1964). Since many entomologists are now focusing on developing new alternative strategies for controlling pests, describing and understanding the exact role and impact of symbionts in *D. radicum* is both a fundamental and applied challenge.

At the start of this PhD, the microbiota of *D. radicum* had just been described from a taxonomic point of view by mass sequencing of 16S DNA, which revealed the presence of *Wolbachia* (Bili *et al.*, 2016). Because it is intracellular, *Wolbachia* is commonly called an "endosymbiont". This bacterium is considered as the most common bacterial symbiont in the animal world, infecting a vast number of insects and other invertebrates around the globe (Werren, 2008). It is known to have a wide range of effects on its host physiology or behavior but nothing had been published yet on the interactions between *Wolbachia* and *D. radicum*. Besides endosymbionts, (extracellular) microorganisms from the gut can also deeply influence life history traits of their hosts (Catania *et al.*, 2017). For this reason, we also studied here the impact of the gut microbiota on some aspects of the biology and ecology of the cabbage root fly (including its relation with its host plant). The particularity of this thesis is that, instead of focusing on one bacterial compartment, we studied different symbionts within a single host (*D. radicum*), by taking both the intracellular *Wolbachia* and gut symbionts into account. Our first study focused mainly

on *Wolbachia*, the second on gut symbionts, while our third study allowed us to discuss about potential links between these two symbiotic compartments.

This thesis is divided into five parts, including three chapters. The first part is a general introduction presenting symbiosis, and two of our study models: the host *D. radicum* and the symbiont *Wolbachia*. In Chapter 1, we describe the effects of *Wolbachia* on some “classical" life history traits of *D. radicum*. In Chapter 2, we present direct (chemical) and indirect effects (loss of gut symbionts) of a tetracycline antibiotic treatment on the life history traits of the cabbage root fly. In this experiment, we use a *D. radicum* population naturally free from *Wolbachia* or other intracellular symbionts, which allows us to isolate the effect of the gut microbiota only. In Chapter 3, we extend our research to a third trophic level: the plant. We first present a brief review of some of the plant chemical defenses against herbivores, and their mechanisms of action. Then, we investigate if a plant reacts differently according to whether the larvae attacking its roots carry *Wolbachia* or not. For this, we identified and quantified volatiles and glucosinolates produced by the plants. Finally, in the last part of this manuscript, we put results obtained during this PhD in perspective, including extra data not presented in the previous chapters, and propose future avenues of research. *In fine*, we present our opinion on the validity of the holobiont concept.

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GENERAL INTRODUCTION

General introduction

1. Symbiosis

1.1. Overview

The term "symbiosis" in its most general – and original – sense was defined by Anton de Bary in 1879 as the intimate "living together of dissimilar organisms", the bigger one being called “host” and the smaller one “symbiont”. Symbioses are widespread in nature and play a major part in the emergence of life forms and diversification of organisms. One of the best illustrations of these phenomenons is the origin of the eukaryotic cell, which emerged after successive symbioses between prokaryotes (Sagan, 1967; Cavalier-Smith, 1992; Gray, 1999). Indeed, it has now been firmly established that mitochondria and plastids, the classical membrane-bound organelles of eukaryotic cells, evolved from bacteria by endosymbiosis. In the case of mitochondria, the evidence points very clearly to an endosymbiont of α-proteobacterial ancestry (Figure 1).

Symbioses affect all levels of biological organization and are vital for virtually every metazoan life form on Earth (Paracer & Ahmadjian, 2000; Moran, 2006). Thus, the evolutionary and ecological significance of symbioses cannot be overestimated.

Like other animals, all insects are colonized by many species of microorganisms which can be located in the gut and within insect tissues, inside and outside their bodies, in a variety of ways (Buchner, 1965; Werren & O’Neill, 1997; Arora & Douglas, 2017). The microbiota of insects contains generally far less species than that of mammals (Engel & Moran, 2013) allowing some microbial functions in insects to be attributed to individual, identified microbial species. This (relative) simplicity of insect symbioses facilitates our understanding of the mechanisms that promote insect-microbial coexistence and the processes by which microbiota affect insect well-being. As a result, insect species which can be bred easily in the laboratory are potentially ideal models to study the various interactions between the host and its resident microorganisms. This work is impractical or unfeasible in mammals, but insect studies can help to generate hypotheses for subsequent testing in mammalian models (Douglas, 2011).

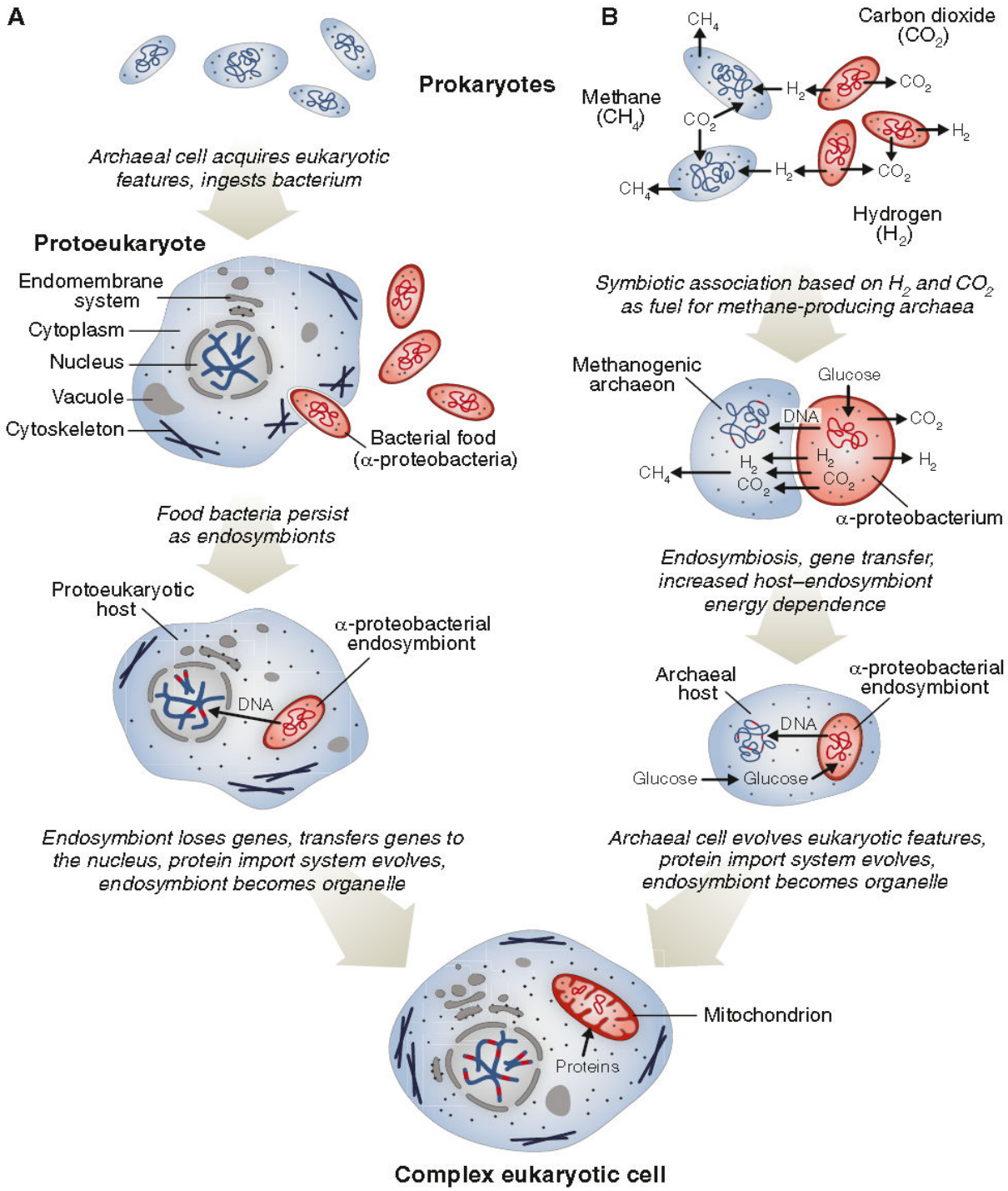


Figure 1. Two competing evolutionary scenarios for the origin of eukaryotic cells and their mitochondria. (A) The traditional view posits that the bulk of eukaryotic cellular complexity arose in a stepwise fashion prior to the endosymbiotic uptake (by phagocytosis) of the α-proteobacterium that became the mitochondrion. (B) The hydrogen hypothesis (Martin & Muller, 1998) invokes a metabolic symbiosis between methane-producing archaea and a-proteobacteria. In this scenario eukaryotic cellular complexity arises after endosymbiosis. Both models involve extensive gene transfer from the a-proteobacterium to the archaeal host and the evolution of a system for targeting nucleus encoded proteins to the endosymbiont-turned organelle. Figure from Archibald, 2015.

We have seen that the definition of symbiosis does not assume that it is a mutually beneficial relationship. Accordingly, symbiont interactions with hosts have been traditionally classified in three artificially clearcut categories: (i) mutualistic (microbe and host mutually benefit each other), (ii) parasitic/pathogenic (the association benefits the microbe but harms the host) or (iii) commensal (the microbe benefits from the host, does no harm but does not provide any advantage either). In reality, assigning a symbiotic association to these groupings is often problematic. For example, mutualism is not maintained on a 50:50 basis: one associate usually takes more, sometimes much more, than the other (Bourtzis & Miller, 2003). More importantly, associations between symbionts and hosts can shift between different states over time and also depend on the phenotypes considered (Clay, 1988; Saffo, 1992). For this reason, a symbiotic relationship should always be defined after careful consideration of the time range, resource conditions, evolutionary adaptations and organisms engaged in the symbiosis (Klepzig *et al.*, 2001). For example, the same organism might act as a beneficial partner in one symbiosis, but as a parasite in another (Aanen & Hoekstra, 2007). The bacterium *Photorhabdus luminescens* is a mutualist or a pathogen depending on whether it inhabits the gut of nematodes or the hemolymph of insects (Ffrench-Constant *et al.*, 2003).

The term "ectosymbiosis" is used when one organism lives on the surface of another, including internal surfaces such as the lining of the digestive tube and the ducts of glands. For example, many invertebrates living in sulfide-rich marine habitats harbor sulfur--oxidizing bacteria on their body surfaces (Dubilier *et al.*, 2008; Goffredi, 2010). The most intimate of symbiotic associations is termed "endosymbiosis", in which one of the partner: the "host", incorporates the other(s) internally. Endosymbionts can occur within their host's cells (intracellular) or outside them (extracellular). Intracellular endosymbionts live directly in the cytoplasm of their host's cells (this is referred to as "endocytobiosis"), while extracellular endosymbionts can be located between the cells of host tissue or in an internal cavity, such as the gut (Bull & Fogarty, 1996).

Terminology in this manuscript: In this work, we will use the words endosymbiont (instead of endocytobiont) to refer specifically to intracellular symbionts such as *Wolbachia,* which live directly within the cells of their hosts. Concerning the community

of symbionts of the gut lumen (which are extracellular), we will use the term "gut microbiota".

Because associations between different microorganisms are extremely diverse (Engel & Moran, 2013; Douglas, 2015; Graf, 2016), I will use the work of Arora & Douglas (2017) to classify symbioses according to two criteria (see Table 1; Arora & Douglas, 2017):

- location of the symbiont (gut lumen or internal tissues such as insect cells);
- mode of transmission (vertical which is from parent to offspring, or horizontal which is from non-parent insects and the external environment).

Table 1. Traits of insect-microbial associations (simplified from Arora & Douglas, 2017).

		Mode of Acquisition	
		Vertical	**Horizontal**
Location	**Gut Lumen**	Maternal fecal pellets consumed by neonatal offspring	Coprophagy, trophallaxis, acquisition of microorganisms in food and water
	Internal (hemolymph, insect cell)	Maternal inheritance via the ovaries	Ingestion and transfer across the gut wall of predators; delivery to prey of parasitoid via ovipositor, during sexual contact

1.2. Symbiont location (gut vs cells)

Symbionts can be located on the body surface of insects, on the surfaces of internal organs or even within the tissues or cells of their hosts. In this work, our main interests are gut microbiota and intracellular symbionts.

Insects experience near constant infection with microorganisms but the bacterial communities vary immensely depending on the species. For example, an adult honey bee contains 10^9 bacterial cells in its gut (Martinson *et al.*, 2012) as well as various endosymbionts depending on the species studied (Yañez *et al.*, 2016), whereas most plant sap-feeding insects contain little (Cheung & Purcell, 1993) or no detectable gut bacteria (Douglas, 1988) but instead harbor high loads of intracellular symbionts (Baumann, 2005). Most insects fall somewhere in between.

In general, a significant proportion of microorganisms reside in the alimentary tract (gut). Microbial communities in the gut are generally open, which means they are subject to invasion by external microorganisms (Engel & Moran, 2013; Douglas, 2015). For this reason, age, environment and diet play a major role in structuring gut microbial communities. Notably, various effects have been observed on the gut microbiota when comparing artificial diets vs. natural foods, as well as between diets in which the major nutritional classes (protein, lipid, sugar, and fiber) are different (Santo Domingo *et al.*, 1998; Kuechler *et al.*, 2011; Tang *et al.*, 2012).

Bacterial endosymbionts are also widespread among invertebrates, particularly among arthropods (Zchori-Fein & Bourtzis, 2011). In this situation, symbionts live within and in close association with their hosts. They form lifelong infections within insects and require their host to survive until sexual maturity and to have a high reproductive output to ensure the endosymbiont persists within the host species over time, since transmission to the next host generation is maternal (Bignell, 2000).

Some intracellular microorganisms are required for survival or reproduction in certain insect groups, where they are restricted to specific cells called bacteriocytes whose sole function appears to be to house and maintain microorganisms (Douglas, 1989). The bacteriocyte symbioses are predominantly closed systems (isolated by location and host factors from incoming microorganisms) (Douglas, 2015).

1.3. Mode of transmission (vertical vs horizontal)

It is well recognized that the way in which symbionts are transmitted plays a crucial role in determining whether parasitism or mutualism will evolve. In the conventional view, horizontal transmission favors parasitism/pathogenicity (Anderson & May, 1982), because then transmission can take place even if the parasite exhausts selfishly the resources of the host and because competition among symbionts rewards the variants which extract the most energy from the host. By contrast, the survival of vertically transmitted symbionts depends on that of their hosts. If symbionts can protect or benefit their hosts, they will bear a selective advantage compared to non-infected individuals, and the symbiont should be maintained within host populations (Lipsitch *et al.*, 1996;

Jones *et al.*, 2007). For this reason, vertical transmission is thought to select for stable coevolutionary relationships between symbiont and host and to evolve towards mutualism which favours an efficient reproduction of the host (Fine, 1975; Ewald, 1987; Yamamura, 1993; Lipsitch *et al.*, 1995). Clearly, if a vertically transmitted endosymbiont and a pathogen both infect the same host, they have different and conflicting interests regarding the survival of the host. However, they can still coexist in a host population, because vertical and horizontal transmission provide different "niches" for the two strains (Lipsitch *et al.*, 1996).

Ewald (1987) proposed that infections with mixed modes of transmission should fall along a continuum, with parasites transmitted mostly horizontally tending toward higher virulence, and agents transmitted mostly vertically tending toward lower virulence. Lipsitch *et al.* (1996) showed that the mode of transmission depends on the selective pressure: horizontal transmission (and higher virulence) will evolve when susceptible hosts are common (high density), whereas vertical transmission (and lower virulence) will evolve when susceptible hosts are uncommon (low density), especially if host fecundity is high. This conflict of interests between different symbionts within a single host is the focus of an emerging area of symbiosis research (Lively *et al.*, 2005).

In insects, gut microbiota can be transmitted horizontally through (i) coprophagy (consumption of feces), (ii) trophallaxis (transfer of food or other fluids among members of a community through mouth-to-mouth “stomodeal” or anus-to-mouth “proctodeal” feeding), or (iii) acquisition of microorganisms from food and water (Arora & Douglas, 2017). However, some species have developed unique ways to transmit their bacteria from mother to offspring (vertical transmission). For example, the plastapid stink bug (*Megacopta punctatissima*) harbors an obligate g-proteobacterial symbiont *Candidatus Ishikawaella capsulata* in the cavities of its crypt-bearing posterior midgut. When adult females lay eggs on their host plant, small brownish particles are always deposited under the egg mass. The particles encase a copious amount of the symbiont, and larvae acquire the symbiont by eating the capsule when hatching from the eggs (Figure 2; Hosokawa *et al.*, 2006; 2007).

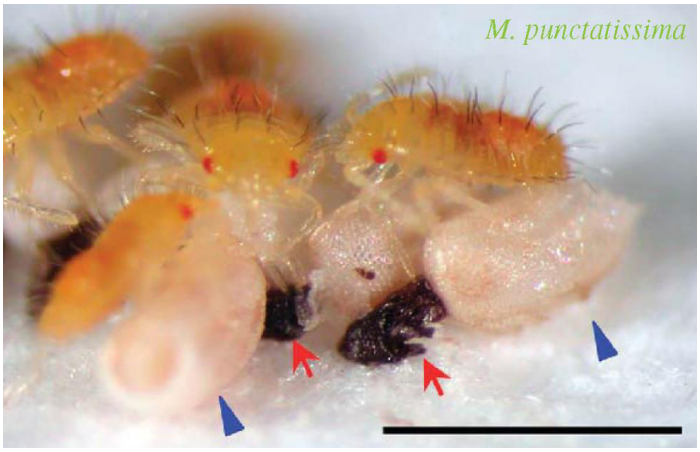


Figure 2. Example of vertical transmission of gut microbiota. Newborn nymphs of *Megacopta punctatissima* probing capsules for symbiont acquisition. Red arrows and blue arrowheads indicate symbiont capsules and eggshells, respectively (picture from Hosokawa *et al.*, 2007).

Contrary to gut microbiota, endosymbionts are most commonly transmitted vertically – from parents to offspring, usually through the females via infection of eggs or embryos, and are thus stably maintained within a host population (Dale & Moran, 2006). However, molecular studies highlight incongruent host and symbiont phylogenies in some associations, where closely related endosymbionts infect phylogenetically distant host species, revealing that these heritable symbionts have undergone occasional horizontal transmission between host species (Sandström *et al.*, 2001; Russell *et al.*, 2003). So far, horizontal transmission of endosymbionts is considered rare and has been difficult to document. However, in aphids, Henry *et al.* (2013) found that not only is horizontal transfer common, but it is also associated with aphid lineages colonizing new ecological niches, including novel plant species and climatic regions. Moreover, aphids that share the same ecologies worldwide have independently acquired related symbiont genotypes, suggesting significant involvement of symbionts in their host's adaptation to different niches. The phylogenies of *Wolbachia* and their hosts are also notoriously incongruent at a large scale, with for example closely related *Wolbachia* found in insects but also in very distantly related woodlice (Isopoda) (Rigaud & Juchault, 1995). Ahmed *et al.* (2015) used fluorescence in situ hybridization (FISH) and multi locus sequence typing (MLST) to reveal evidence for the horizontal transmission of *Wolbachia* between insect hosts by parasitic wasps, via their mouthparts and ovipositors. Another suspected route of horizontal transmission of *Wolbachia* was predation and cannibalism. In 2013, Le Clec'h *et al.* made the first demonstration of *Wolbachia* occurrence in various organs of an

initially uninfected host after eating an infected one. Indeed, after eating *Armadillidium vulgare* harboring *Wolbachia*, *Wolbachia*-free woodlice predators were tested positive for the presence of the symbiont.

1.4. Extended phenotype of the host

Bacterial symbionts are diverse and widespread among insects and exhibit diverse effects on their hosts, which can be beneficial or detrimental (Oliver *et al.*, 2010; Zchori-Fein & Bourtzis, 2011). It is now widely recognized that the union of symbiont and host provides opportunities for the evolution of novel phenotypes, by combining genomes with different biochemical capabilities (Werren & O'Neill, 1997). Bacterial symbionts are thus "hidden players" in many ecological and evolutionary processes (Frago *et al.*, 2012).

The gut microbiota is very well-known to upgrade nutrient-poor diets (Flint, 2012; Wong *et al.*, 2014) or aid digestion of recalcitrant food components (Brune, 2014). Moreover, gut symbionts have also been shown to protect from pathogens (Dillon *et al.*, 2005); detoxify plant compounds (Despres, 2007); influence tissue development (Shin *et al.*, 2011) or even circadian regulation (Leone, 2015). More recent studies found that gut microorganisms also contribute to inter and intraspecific communication, can govern mating and reproductive systems (Archie & Tung, 2015; Morimoto *et al.*, 2017; Walsh *et al.*, 2017), and even drive speciation (Brucker & Bordenstein, 2013).

Endosymbionts also have extremely various effects on their hosts depending on the host-symbiont couple studied. Increasingly, experimental evidence shows that some vertically transmitted symbionts protect their hosts against pathogens or predators, a process described as "symbiont-mediated protection" (Oliver *et al.*, 2005). For example, symbionts of aphids can increase their resistance to parasitoid wasps (Oliver *et al.*, 2003; Oliver *et al.*, 2005; Vorburger *et al.*, 2009 ; Heyworth & Ferrari, 2015; Donald *et al.*, 2016) or fungi (Scarborough *et al.*, 2005; Łukasik *et al.*, 2013; Heyworth & Ferrari, 2015). Endosymbionts can also influence "classical" life-history traits such as fecundity and body mass (Castañeda *et al.*, 2010; Vorburger *et al.*, 2013), longevity (Fukatsu *et al.*, 2001; Polin *et al.*, 2014), body color (Tsuchida *et al.*, 2010), growth or reproduction (Fukatsu *et al.*, 2001). Intracellular bacteria can also influence the nutrition supply of their hosts (Koga

et al., 2003; 2007), their resistance to heat shock (Chen *et al.*, 2000; Russell & Moran, 2006; Guay *et al.*, 2009; Heyworth & Ferrari, 2015) and more surprisingly, behavioral traits such as defensive behaviour (Polin *et al.*, 2014).

1.5. Obligate (primary) and facultative (secondary) symbiosis

When symbiosis is indispensable for the survival of the host, symbionts are called "obligate" or "primary" and hosts entirely depend on them for survival, development or reproduction (Moran, 2006; Guo *et al.*, 2017). For example, in aphids, the obligate symbiont *Buchnera aphidicola* is vital for aphids as it provides them essential amino acids they cannot obtain themselves from the phloem of plants (Baumann, 2005; Douglas, 1998; Shigenobu *et al.*, 2000). In some cases, heritable bacterial symbionts such as *Buchnera* in aphids and *Wigglesworthia* in tsetse flies are housed in specialized host organs called bacteriomes (Figure 3). This adaptation has inextricably linked the evolutionary fates of symbiont and host and thus led to a strictly obligate relationship, and an evolution of mutualism between them (Zug & Hammerstein, 2015).

When the symbiont is not required for the successful development or reproduction of the host, symbionts are called "facultative" or "secondary" symbionts (S-symbionts). Examples include a large number of insect associates such as *Wolbachia pipientis*, *Spiroplasma sp.*, and facultative symbionts of aphids and relatives (Moran, 2006).

Finally, some symbioses, such as the association between the bacterium *Vibrio fischeri* and certain squid and fish species, appear to be largely obligate for both parties but also entail a life cycle stage in which the symbiont replicates outside the host. These distinctions make a very large difference for the genome evolution of the symbiont and, in turn, for the biology of the host (Moran, 2006).

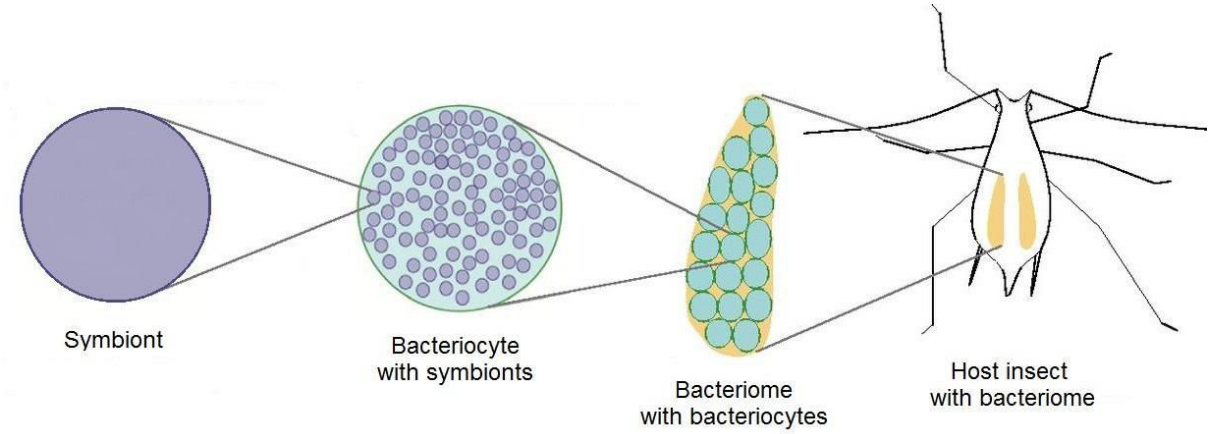


Figure 3. Organization of endosymbionts in insect hosts. Insect endosymbionts (purple) are housed into bacteriocyte cells which can contain thousands to tens of thousands of symbionts. Many bacteriocyte cells aggregate to form an organ called the bacteriome. Bacteriomes are typically paired in insects (modified from Wilson & Duncan, 2015).

Most commonly, "obligate" or "facultative" symbiosis refer to the host's "point of view", i.e. whether the host needs or not the symbiont to survive. From the symbiont's perspective however, the association is most often obligate. Indeed, most microorganisms in natural communities are likely to have obligate dependencies on other species (often other microbes), explaining why 99% of microorganisms are difficult or impossible to culture (Sapp, 2004).

1.6. Symbionts and evolution

Given the many examples of symbiosis known today with diverse physiological and morphological effects (some beneficial, others detrimental for the host), it is clear that symbiosis plays an important role in development and heredity and that it is a general mechanism of evolutionary innovation (Maynard Smith, 1989; Margulis & Fester, 1991; Siozios *et al.*, 2018). Symbiosis could indeed be one of the most effective mechanisms of biological innovation.

According to the contemporary conceptual consensus, the mitochondria of all eukaryotic cells and the chloroplasts of plants and protists were once free-living bacteria (alpha-proteobacteria and cyanobacteria, respectively) that became incorporated in a primitive

host cell (Margulis, 1998; Gray, 1999). Even the cell nucleus may have also arisen by some sort of fusion of symbiosis between two different kinds of bacteria (Sapp, 2004).

Multicellular organisms such as ourselves did not just evolve from bacteria-grade unicellular ancestors; we were "upgraded" by bacterial symbiosis and have maintained a close association with them ever since. When sequencing an organism, the DNA obtained actually comes from two sources: first, the standard nuclear and mitochondrial genomes of the organism host; second, sequences from heritable microbes — bacteria that pass from a mother to her progeny and represent part of the "extended genome" of the host (Hurst, 2017).

Even though eukaryotes are the most morphologically complex organisms, microorganisms represent the largest biomass on earth (Whitman *et al.*, 1998) and have the greatest biochemical complexity. Indeed, microbial symbionts carry out many chemical reactions otherwise impossible for their hosts to perform (i.e. they can photosynthesize, fix nitrogen, metabolize sulphur, digest cellulose, synthesize amino acids, provide vitamins and growth factors, and ward off pathogens; Sapp, 2004).

Accordingly, just like genetic mutations, the acquisition of bacterial symbionts can be perceived as a source of inheritable genetic variation via the symbiotic genome(s), which can influence the phenotype of their hosts. Indeed, the acquisition of a symbiont in a natural population can be assimilated to a whole set of genetic "mutations" with the extraordinary bonus that none of the functional genes of the host is altered in the process: it is more exactly a massive addition of genetic information (Ferrari & Vavre, 2011). Indeed, the symbiont brings at once a whole array of completely new "ready-to-use" functions (that can be beneficial or not for the host), and thus can sharply steer the evolutionary trajectory of its host species. These shared effects on phenotypes then allow to link population genetics models to theoretical models of symbiotic associations (Leclair, 2016).

To go further, Olivieri (2010) proposed that despite its intrinsic complexity, an inheritable bacterial symbiont fundamentally behaves like a diallelic locus in a haploid host population: the first allele corresponds to the presence of the symbiont (infected host) and the second allele, its absence (uninfected host). However, this analogy is only valid for facultative heritable symbionts since obligate symbionts are fixed in host

populations while hosts will be polymorphic for facultative symbionts. The frequencies of these facultative symbionts will then be modulated by the classical evolutionary forces regulating allelic frequencies (Oliver *et al.*, 2010), i.e. mutations, natural selection, genetic drift and migration/gene flow (Figure 4).

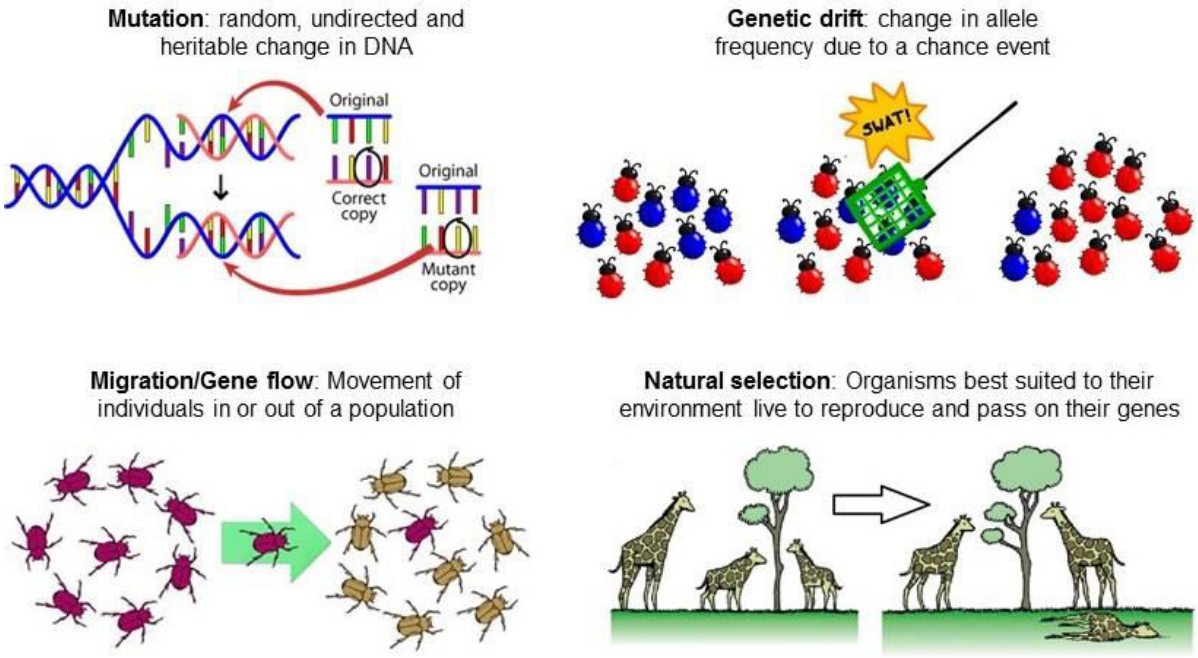


Figure 4. Representation of the four main evolutionary forces in population genetics.

To dissect the idea that the holobiont is a unit of selection, Hurst (2017) proposed a simplified model of natural selection and adaptation. For a standard trait, this can be stated as three 'ifs' and a 'then':

- if there is phenotypic variation in a character, and
- if phenotypic variation is associated with fitness variation (variation in the probability of survival/ fertility between individuals), and
- if the phenotype is at least partially heritable,

then, the composition of the next generation will be biased with respect to heritable factors which increase the probability of survival/fertility.

If this model is applied to bacterial symbionts, we obtain:

- if symbionts vary between hosts, producing phenotypic variation, and
- if this variation influences host fitness, and
- if some symbionts are heritable,

then the constitution of the microbiota is under selection, and can be considered as a functional part of the host.

Accordingly, there is a real need to document host-microbe interplay to establish the evolutionary ecology and dynamics of host–microbe interactions.

In this thesis, we will try to unravel the relationships existing between the cabbage root fly (*Delia radicum*), a belowground herbivore developing on brassicaceous plants, and (i) *Wolbachia*, an intracellular symbiont that has been recently detected in wild populations (Bili *et al.*, 2016), (ii) its gut microbiota.

2. The host: the cabbage root fly

2.1. Taxonomy and distribution

The cabbage root fly *Delia radicum* (Diptera: Anthomyiidae) presents several synonyms in the literature, such as *Hylemyia brassicae* (Bouché)*, Chortophila brassicae* (Bouché), *Delia brassicae* (Wiedemann) and *Erioischia brassicae* (Bouché) (Miles, 1950; Doane & Chapman, 1964; Zohren, 1968; Ryan & Ryan, 1980).

This species is distributed in all the northern holarctic region, from Canada to Russia, and Europe (CABI, 1989). It was accidentally introduced in North America in the 1800s, possibly as pupae in soil used as ship ballast (Schoene, 1916). Analysis of egg micromorphology and DNA variation among different populations indicate a single introduction to eastern North America from northwestern Europe, followed by the spread of the founder population westward across the rest of North America (Biron *et al.*, 2000; 2003). In Canada, *D. radicum* is generally restricted to agricultural landscapes, and absent in more natural areas (Griffiths, 1991).

2.2. Identification

D. radicum imagos are about 6 mm long and are very similar to the house fly (*Musca domestica*). Several morphological traits allow to easily distinguish males and females in the laboratory. Males have contiguous eyes (Figure 5a), their body is generally dark grey and covered with bristles (Figure 5b) and their abdomen is thin, pointy and black (Figure 5c), whereas females have widely separated eyes (Figure 5d), a lighter body with bristles (Figure 5e), and an abdomen larger and wider than that of males (Figure 5f) (Smith, 1927) (Figure 5).

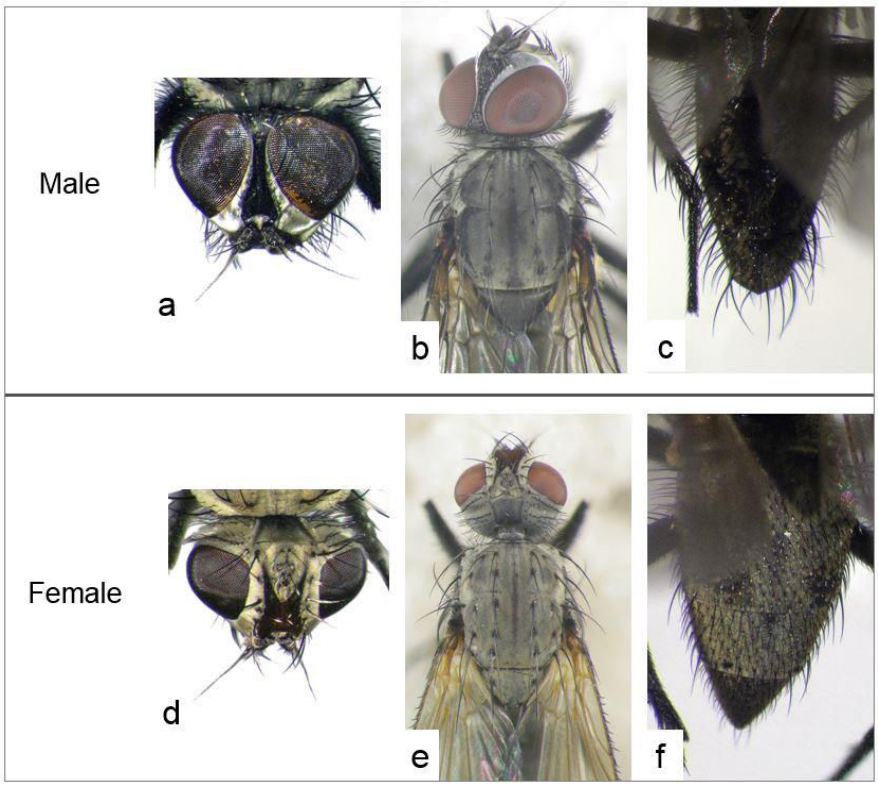


Figure 5. Morphological criteria to distinguish *Delia radicum* males and females (pictures by Sonia Dourlot).

Cabbage root fly eggs have the appearance of tiny grains of rice, and measure on average 0.93 to 1.02 mm (Coaker & Finch, 1971) (Figure 6a). Their incubation varies from 2 to 14 days and depends on the temperature and humidity (De Wilde, 1947; Neveu *et al.*, 1997). When the temperatures are between 15°C to 20°C, eggs will hatch on average 4 to 6 days after being laid. In the lab, females can lay up to 375 eggs while in the field, this number usually drops around 60 to 80 eggs on average (Swailes, 1961; Finch & Coaker, 1969; Coaker & Finch, 1971).

Larvae are maggots (Figure 6b), pass through three larval stages and grow from 1 mm long when they hatch to 8 mm long and 1-2 mm in diameter at the end of their L3 stage (Smith, 1927). Pupae measure 5-8 mm, are brownish and possess on their posterior end two easily recognizable tubercules (Brooks, 1951) (Figure 6c). The weight and size of the pupae depend on the quantity (Hopkins *et al.*, 1999) and quality (Soler *et al.*, 2007a) of the available food during their larval development.



Figure 6. *Delia radicum* egg, larva and pupa (pictures from Sonia Dourlot).

2.3. Biology and ecology

The cabbage root fly is a holometabolous insect, which means that the adult (or imago) is morphologically completely unlike the larval stages and emerges from the immobile pupal (nymphal) stage following a dramatic remodeling of the whole body.

In optimal climatic conditions (20 ± 1° C, 60 ± 10% RH) with food *ad libitum*, *D. radicum* develops in 30 days (Figure 7). The mated female lays eggs at the collar of host plants. Females can lay approximately 150 eggs during their lifespan. After hatching, larvae dig galleries in the root of the plant while feeding. In these climatic conditions, the first instar lasts 3 days, the second 4-5 days, and the third 16-17 days (Coaker & Finch, 1971). L3 larvae migrate at about 8-12 cm depth in the soil near the roots to pupate. Adults emerge about ten days later (Finch, 1989) (Figure 7). In this species, males emerge on average 2.5 days before females. This very common phenomenon in insects is called protandry and allows precocious males to wait and be first to mate with emerging females from the same patch (Godfray, 1994), which increases their chance of paternity (Simmons et *al.*, 1994). Once adults, females have quite a short lifespan (12 to 15 days at 20°C) but some individuals and males can live up to 4-6 weeks (Read & Brown, 1966; personal observations).

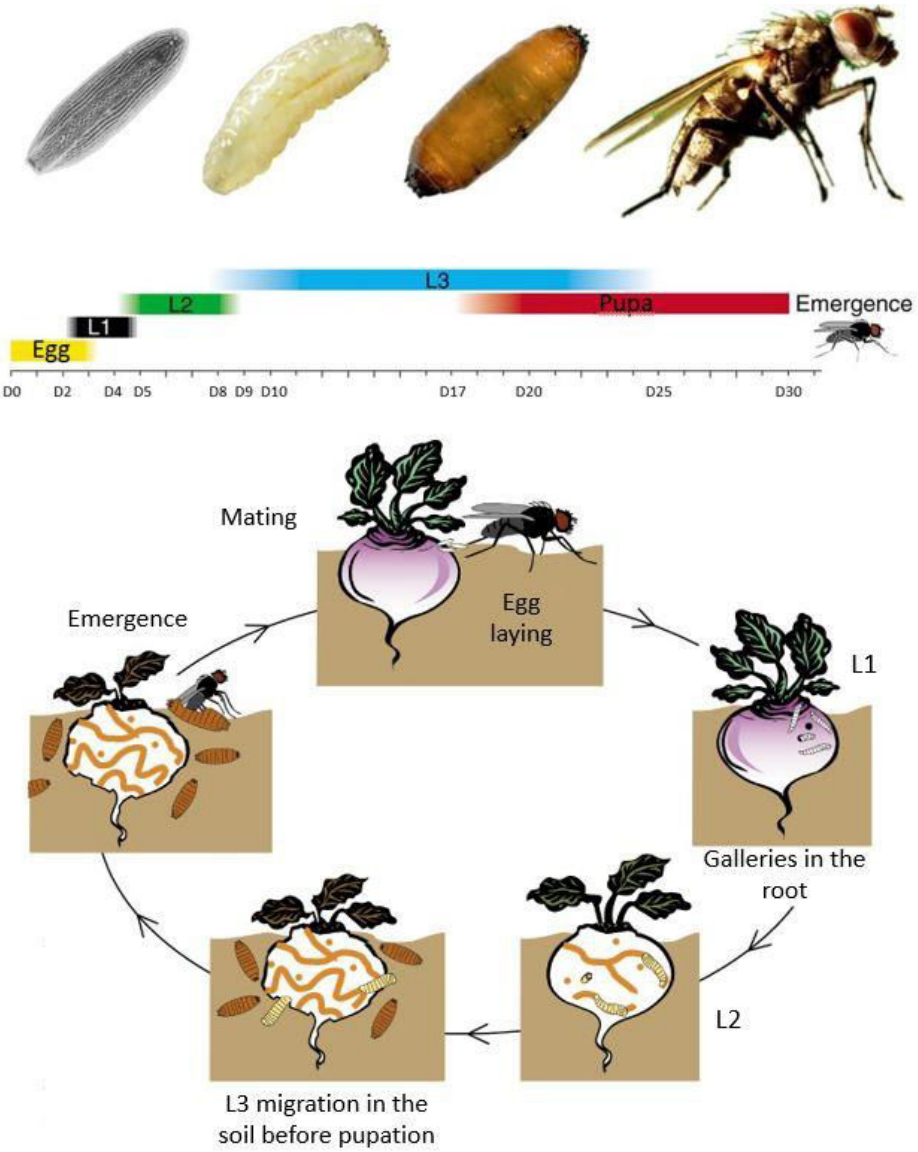


Figure 7. *Delia radicum* development cycle (illustration by Sonia Dourlot). After mating, females lay their eggs in the soil in the immediate vicinity of plants. After hatching, L1 larvae dig galleries in the root of the plant while feeding, and grow up to become L2 larvae. Then, L3 larvae migrate in the soil near the roots to pupate. Finally, male and female adults emerge, mate again, and a new cycle begins.

The cabbage root fly is an oligophagous insect specialist of the Brassicaceae family. It is an economically important pest of brassicaceous crops within the temperate zone of the holarctic region (Finch, 1989). In untreated crops, *D. radicum* damage typically causes a 25% yield loss, but it can reach 90% for cabbage or turnip (King & Forbes, 1954; Skinner & Finch, 1986; Söndgerath & Miller-Pietralla, 1996). The fly infests all types of cultivated Brassicae, including rutabaga, turnip, cabbage, cauliflower, broccoli, kale, kohlrabi, Brussel sprouts, collards, mustard, oilseed rape, radish, and horseradish (Griffiths, 1991).

However, the fly developmental success varies a lot depending on the plant species/variety (Doane & Chapman, 1962; Finch & Ackley, 1977; Ellis *et al.*, 1999; Hopkins *et al.*, 1999; Felkl *et al.*, 2005; Shuhang *et al.*, 2016).

2.4. Interactions with its host plant

Females lay their eggs in small clusters in the immediate vicinity of stems of Brassicaceae plants (Figure 8). Before they have the chance to fully develop, a large part of these eggs and larvae will be predated by several species of carabidae and staphylinidae (Wishart *et al.*, 1956; Hugues, 1959; Coaker & Williams, 1963; Wright *et al.*, 1960; Mowat & Martin, 1981). Larvae dig galleries inside the roots while feeding, leading to root decay when infestation is heavy (Griffiths, 1986) since the lesions on the roots provide a point of entry for subsequent infection by endemic root rot pathogens (Soroka, 1997).



Figure 8. *Delia radicum* ovipositing clusters of eggs at the collar of a plant (picture by Sonia Dourlot).

Even though larvae feed on roots, gravid females use above-ground signals from the leaves to decide where to lay their eggs (Finch & Collier, 2000). Host finding and oviposition follow several steps. First, females search by flight for the plant volatiles that will stimulate their activity and their egg-laying (Finch, 1978; Wallbank & Wheatley, 1979). Upon detecting them, they make the decision of landing on a particular plant according to several criteria such as leaf size, color and pattern, leaf structures, plant height above ground, substrate color and visual prominence (Prokopy *et al.*, 1983; Tuttle *et al.*, 1988; Roessingh & Städler, 1990). Once on the plant, females explore it with contact

chemoreceptor sensillae located on their tarsus to detect specific leaf surface compounds such as glucosinolates that will stimulate their descent towards the ground to lay eggs (Roessingh *et al.* 1992; 1997). Only when all their criteria are validated will they climb down the stem, probe the soil, and oviposit at the base of the root collar (Zohren, 1968).

When *D. radicum* begins to chew on a root, there is a biochemical cascade of events that precedes changes in plant physiology. Indeed, plants have evolved receptors to detect specific insect elicitors present on their cuticle or in their oral secretions or saliva, resulting in the activation of plant defense pathways (Felton & Tumlinson, 2008). However, the precise elicitors of *Brassica* defense against *D. radicum* have not been identified yet, although the defense reaction itself is well known.

First, herbivory by *D. radicum* modifies the levels of primary and secondary non-volatile compounds used in direct defense (Hopkins *et al.*, 1999; van Dam & Raaijmakers, 2006; Pierre *et al.*, 2012), such as glucosinolates (GSLs). In wild *Brassica* species, *D. radicum* feeding on roots increases the levels of indole and aliphatic glucosinolates (GLS) produced by the plant both locally (in roots) and at a systemic level (in shoots) (van Dam & Raaijmakers, 2006). When *D. radicum* larvae chew on *Brassica* tissues, these glucosinolates (which are not volatile) get converted into toxic degradation compounds (isothiocyanates, thiocyanates, indoles…) that larvae have to detoxify when feeding (Vig *et al.*, 2009).

Second, herbivory can induce the emission of volatile organic compounds by the plant, called Herbivore Induced Plant Volatiles (HIPVs). HIPVs are specific to herbivore damage: they differ from volatile compounds elicited by mechanical damages or released by intact plants (Turlings *et al.*, 1995; Takabayashi & Dicke, 1996). More specifically still, plants can recognize which herbivore species is feeding on them and adapt their response accordingly. Therefore, for a single plant species, the plant response may vary according to herbivore identity, leading to the production of specific qualitative and quantitative mixtures of volatile compounds (Dicke *et al.*, 2009). This plant ability provides *D. radicum* natural enemies - such as parasitoids - with a reliable cue to locate their host or prey (Neveu *et al.*, 2002; Soler *et al.*, 2007b; Pierre *et al.*, 2011).

3. The symbiont: *Wolbachia*

3.1. Taxonomy and distribution

Wolbachia were discovered almost a century ago in the reproductive tissue of the mosquito *Culex pipiens* (Hertig & Wolbach, 1924), and the species *Wolbachia pipientis* was formally described a few years later (Hertig, 1936). *Wolbachia* are alpha-Proteobacteria (gram negative) members of the order Rickettsiales, a diverse group of intracellular bacteria that live in the cytoplasm of cells. Unlike members of related genera such as *Anaplasma*, *Ehrlichia* and *Rickettsia*, *Wolbachia* do not routinely infect vertebrates. Instead, they have a strict association with invertebrates (Anderson & Karr, 2001). *Wolbachia* are the most abundant endosymbiotic bacteria worldwide, infecting a major proportion of arthropods (Hilgenboecker *et al.*, 2008; Zug & Hammerstein, 2012, 2015; Martinez *et al.*, 2016). Recent statistical estimates suggest that up to 52% of species may be infected with one or more strains of this microbe (Hilgenböcker *et al.*, 2008; Weinhert *et al.*, 2015). Notably, they are found in species from all major insect orders, in particular the most species-rich groups Coleoptera (beetles), Diptera (flies), Hymenoptera (ants, bees, wasps, and sawflies), and Lepidoptera (moths and butterflies). Besides insects, *Wolbachia* have been found in every arthropod subphylum (Werren, 2008; Mock *et al.*, 2016) and also, in aquatic hosts (Sontowski *et al.*, 2015).

Several variants of Wolbachia exist and they are classified in several groups (Figure 9). There were two mains groups (A and B) in arthropods which are termed “supergroups” (Werren *et al.*, 1995; Lo *et al.*, 2007). Those two clades have at least eight potential groups within A and four within B (Zhou *et al.*, 1998). Other supergroups have also been described, including *Wolbachia* infecting nematodes (C and D supergroups) (Bandi *et al.*, 1998), supergroup E in Collembola (Vandekerckhove *et al.*, 1999; Czarnetzki & Tebbe, 2004), F in arthropods and nematodes (Casiraghi *et al.*, 2005), G in spiders (Rowley *et al.*, 2004) and H in termites (Bordenstein & Rosengaus, 2005).

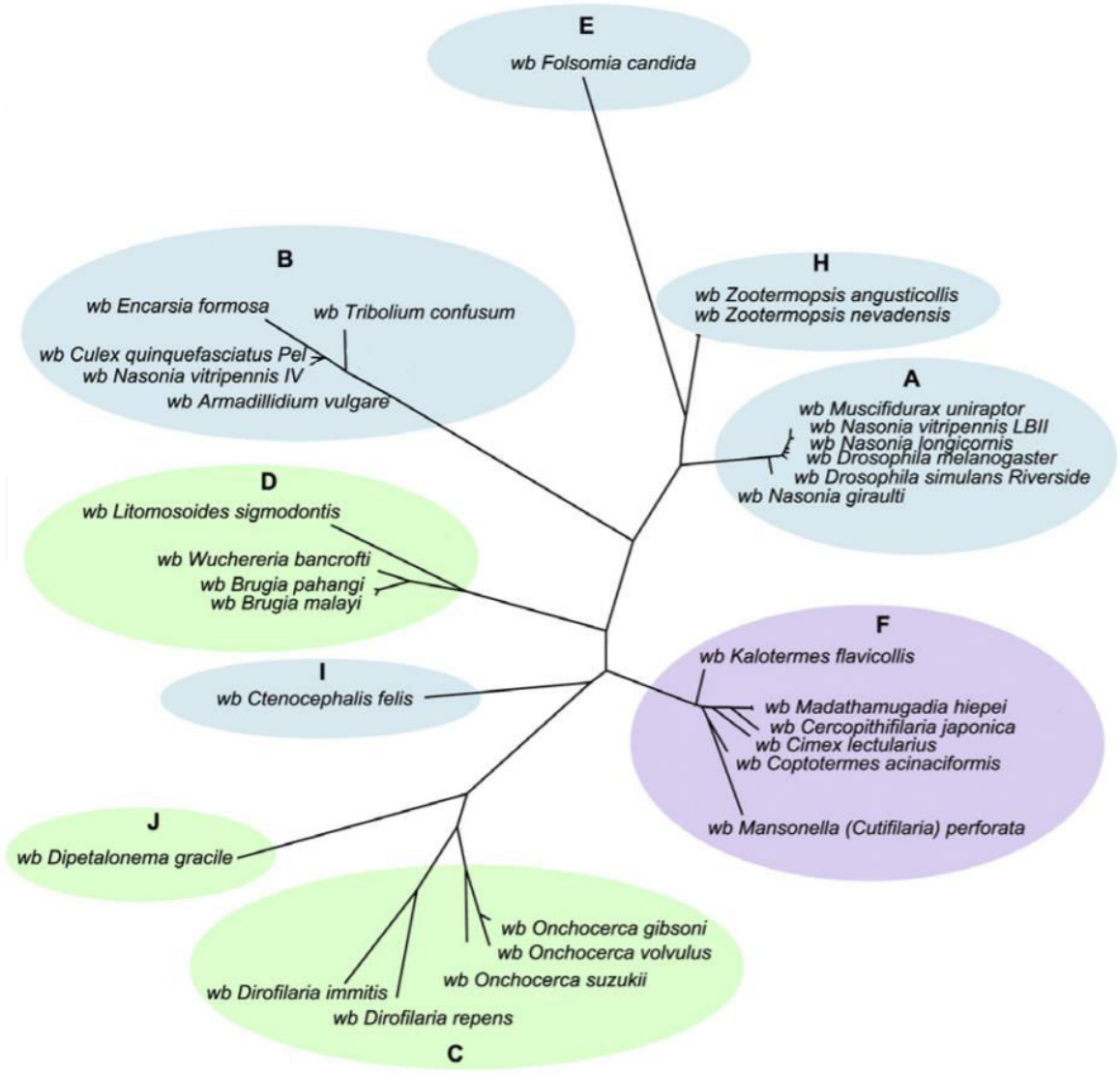


Figure 9. Unrooted phylogeny of *Wolbachia* based on concatenated datasets of 16S rDNA, groEL, ftsZ, dnaA and coxA sequences. From Lefoulon *et al.*, 2012.

3.2. Vertical transmission

Wolbachia are predominantly transmitted vertically, through the eggs of infected females (Werren, 1997). In order to be successful, this bacteria thus needs to be transmitted as efficiently as possible. In nature, the efficiency of vertical transmission is very variable (Hoffmann *et al.*, 1996; Jiggins *et al.*, 2002). In any case, *Wolbachia* need to be located in the female germline to be transmitted to the eggs. To do so, the bacteria has developed several strategies which ensure its presence in the germline stem cells and thus access to the developing egg (Serbus *et al.*, 2008).

3.3. Reproductive manipulations

In addition to their efficient maternal transmission, many *Wolbachia* bacteria also increase their prevalence in populations by manipulating host reproductive systems (O'Neill *et al.*, 1997). All these reproductive manipulations enhance the proportion of infected females and thus benefit the maternally inherited *Wolbachia* (Werren *et al.*, 2008). This symbiont has evolved intriguing ways to interfere with key reproductive processes of its arthropod hosts, all of which enhance the proportion of infected females and thus benefit the maternally inherited *Wolbachia*. These reproductive phenotypes are known as feminization (FI), parthenogenesis induction (PI), male-killing (MK) and cytoplasmic incompatibility (CI), respectively, and are collectively referred to as "reproductive parasitism" (Hurst & Frost, 2015) (Figure 10). Indeed, regardless of how *Wolbachia* manipulate host reproduction, the result improves their own transmission; therefore they commonly serve as examples of "selfish genetic elements" (SGEs)[1] (Werren, 2011). Even though these phenotypes can be induced by other maternally inherited bacteria such as *Arsenophonus*, *Cardinium*, *Rickettsia* or *Spiroplasma* (Engelstädter & Hurst, 2009), *Wolbachia* are special in that they are the only microbes known so far to induce all four phenotypes. Because these manipulations are sufficient to ensure their spread, the endosymbiont can afford to decrease host fitness. This strategy thus represents an alternative route to persist in hosts, without evolving towards mutualism (Werren, 2011).

3.3.1. Sex ratio distorters

Three of these reproductive phenotypes (FI, MK and PI) distort the offspring sex ratio of infected mothers towards females and thus directly increase the proportion of infected females. In doing so, these manipulations create a potential genetic conflict over sex

[1] Some genes (or larger genetic elements) within an individual's genome act to further their own evolutionary interests at the expense of the individual as a whole, which puts these elements into conflict with the rest of the genome. A particular type of genetic conflict arises between nuclear and cytoplasmic elements, deriving from the difference in their inheritance patterns. While nuclear genes are typically inherited through both sexes, cytoplasmic elements, such as mitochondria, chloroplasts, and most heritable endosymbionts, are generally transmitted maternally only, that is, through the cytoplasm of the egg. This results in a cytonuclear conflict, in particular over sex determination and sex ratios.

determination, which can result in rapid evolution of a sex-determining system (Cordaux *et al.*, 2011).

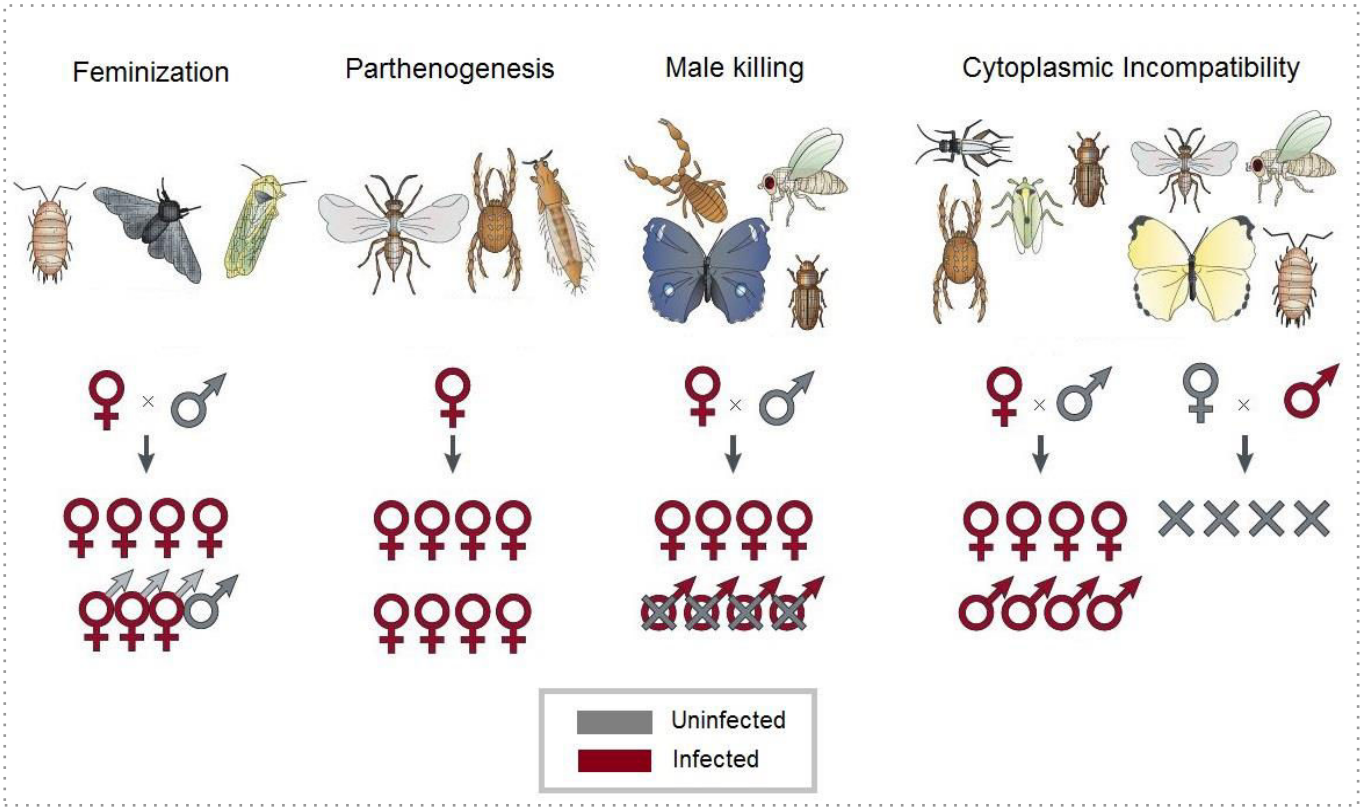


Figure 10. *Wolbachia*-induced phenotypes (modified from Werren *et al.*, 2008). *Wolbachia* cause four distinct reproductive phenotypes in a range of arthropod orders. Feminization results in genetic males that develop as females (in the Hemiptera, Isopoda and Lepidoptera orders). Parthenogenesis induction eliminates males from reproduction (in the Acari, Hymenoptera and Thysanoptera orders). Male killing eliminates infected males to the advantage of surviving infected female siblings (in the Coleoptera, Diptera, Lepidoptera and Pseudoscorpiones orders). Cytoplasmic incompatibility prevents infected males from successfully mating with females that lack the same *Wolbachia* types (in the Acari, Coleoptera, Diptera, Hemiptera, Hymenoptera, Isopoda, Lepidoptera and Orthoptera orders).

In the feminization phenotype, infected but non-transmitting male embryos develop as *functional* females, which do transmit the infection. FI occurs through different mechanisms in different hosts and is not restricted to a specific sex determination system (Bouchon *et al.*, 2008). An important evolutionary outcome of this phenotype in female-heterogametic hosts (e.g. ZW) is the elimination of the female sex chromosome (W). The reason is that feminized ZZ individuals produce functional females without transmitting

the W chromosome. This is an example of cytonuclear conflit as the sex ratio is not determined by the chromosomes anymore (nuclear sex determination) but by the presence/absence of the feminizer symbiont (cytoplasmic sex determination) (Cordaux *et al.*, 2011).

Induction of thelytokous parthenogenesis has so far been found only in haplodiploid host taxa. Here, *Wolbachia* induce unfertilized eggs, which would normally develop into haploid males (arrhenotoky), to develop into diploid females (thelytoky), thus again increasing the percentage of transmitting hosts (Stouthamer *et al.*, 1990). Though more convenient, labeling this reproductive manipulation as "parthenogenesis induction" is, strictly speaking, not correct as arrhenotoky already constitutes a form of parthenogenesis. *Wolbachia* actually cause diploidization of unfertilized eggs to give birth to females. Two mechanisms have been observed: (i) gamete duplication, where meiosis is normal, but diploidy is restored by disruption of the cell cycle during early embryonic development (Gottlieb *et al.*, 2002), and (ii) meiosis failure in *Wolbachia*-infected eggs resulting in diploid gametes (Weeks & Breeuwer, 2001). *Wolbachia*-induced thelytoky can have an interesting evolutionary implication in populations where infection has gone to fixation. In such all-female populations, the loss of sexual traits makes females dependent on their symbionts for daughter production (Zug & Hammerstein, 2015).

In the male-killing phenotype, *Wolbachia* kills male offspring of infected females. This killing occurs during embryogenesis, which results in more food for the surviving female progeny, especially when they eat their dead brothers (Werren *et al.*, 2008). The logic behind MK is different from the two previous phenotypes as the transmission rate do not increase through females *per se*. Instead, daughters benefit from the death of their brothers through some form of fitness compensation (i.e. kin selection theory): (i) reduced competition between siblings, (ii) reduced inbreeding, and (iii) resource reallocation, e.g. through sibling egg cannibalism (Hurst, 1991). Different mechanisms can induce MK and one of them seems to occur through lethal feminization (Kageyama & Traut, 2004). Some defects caused by MK are highly similar to the ones observed in cytoplasmic incompatibility, pointing to a similar mechanism underlying both phenotypes. Interestingly, suppression of the MK phenotype does not necessarily eliminate *Wolbachia*'s ability to induce MK. When *Wolbachia* that induce CI in their native host *Drosophila recens* were introgressed into *D. subquinaria*, they caused MK in the novel

host (Jaenike, 2007). Therefore, *Wolbachia* can maintain their MK ability despite host resistance, and actually can induce two distinct phenotypes, CI and MK, again indicating a similar underlying molecular basis.

3.3.2. Cytoplasmic incompatibility

Cytoplasmic incompatibility (CI) is the most common *Wolbachia*-related reproductive manipulation. In this case, the fitness of uninfected females is reduced by making them incompatible with infected males, which increases indirectly the fitness of infected females (that are compatible with all males). CI can be unidirectional when the male is infected but the female is not. However, the opposite cross is compatible (infected female x uninfected male) as well as mating between two infected individuals. Bidirectional incompatibility happens when the individuals do not carry the same *Wolbachia* strain. In this case, embryonic mortality occurs in both directions (Charlat *et al.*, 2001). This can lead to a postzygotic reproductive isolation between both types of individuals, which will reduce genetic exchanges between them. For this reason, bidirectional CI could be a potential speciation factor (Werren, 1997).

In haplodiploid species, CI does not always lead to the death of embryos. In the *Nasonia* genus, mortality is observed in *N. longicornis* and *N. giraulti*, but CI induces conversion to male development (haploid) in *N. vitripennis* wasps (Tram *et al.*, 2006).

3.4. Effects on the host phenotype

In the earlier works following its discovery, *Wolbachia* was only considered as a reproductive parasite because of the reproductive manipulations it can cause on its host (Werren *et al.*, 2008). However, recent years have witnessed the rapid accumulation of evidence that some *Wolbachia* strains can also benefit their hosts while other are clearly pathogenic and can severely shorten the longevity of their host (Min & Benzer, 1997).

Some of the detrimental effects *Wolbachia* can cause can be considered as “side effects” of the reproductive manipulation described earlier. For example, male-killing *Wolbachia* reduce the fitness of infected females by killing some of their offspring (Jiggins *et al.*,

2002). Feminizing *Wolbachia* also impose a fitness cost on infected neo-females (i.e. feminized males) in that males prefer genetic females over neo-females, which have lower mating rates and receive less sperm (Moreau *et al.*, 2001). But *Wolbachia* can also be detrimental to their host without manipulating its reproduction. Notably, infection has been found to be associated with reduced fecundity of female hosts (Hoffmann *et al.*, 1990; Stouthamer & Luck, 1993; Perrot-Minnot *et al*., 2002). An unusual *Wolbachia* strain has also been described, called *w*Dm*popcorn*, which severely reduces *Drosophila melanogaster* survival. This strain massively proliferates in the adult, causing widespread degeneration of tissues, including brain, retina, and muscle, culminating in early death (Min & Benzer, 1997).

However, even for reproductive parasites, it can pay to enhance host fitness. Indeed, there is a recent upsurge of reports on *Wolbachia*-associated fitness benefits (Zug & Hammerstein, 2015). Turelli (1994) showed that selection on CI-inducing *Wolbachia* favours variants that increase the relative fecundity of infected females, even if these variants reduce the strength of CI. Vavre *et al.* (1999) also showed that in the parasitoid wasp *Trichogramma bourarachae*, *Wolbachia* increases fecundity without any evident reproductive manipulation ("stand-alone benefit" infection). During the last few years, numerous studies have reported that *Wolbachia* infection has an anti-pathogenic effect in the host, for example against several RNA viruses, different *Plasmodium* species, fungi, bacteria, and nematodes (Zug & Hammerstein, 2015). For example, in *Drosophila*, they provide protection against the highly lethal fly virus *Drosophila C virus* (DCV) (Martinez *et al.*, 2016). In the bedbug, *Wolbachia* even reached the status of obligate symbiont and resides in a special organ, the bacteriome, where it supplies its host with B vitamins (Hosokawa *et al.*, 2010). A recent study also suggests that *Wolbachia* increase lifetime reproductive success of the wasp *Anagrus sophiae* in the field (Segoli *et al.*, 2013).

Any fitness benefit conferred by facultative symbionts is highly dependent on the environment and variable among host genotypes (Correa & Ballard, 2016). A study by Fry and Rand (2002) evaluated the effects of the wMel strain infection in laboratory maintained *D. melanogaster*, and found that infected flies lived longer, the effect being highly dependent on their genetic background. Brownlie *et al.* (2009) also found direct evidence on the beneficial role of *Wolbachia* in the iron metabolism of *D. melanogaster*. Indeed, the symbiont increased the fecundity of females during periods of nutritional

stress – when subjected to low or high iron environments. Based on the seemingly low iron content of flies in nature, the authors concluded that *Wolbachia* contribution to the host iron metabolism is very likely an ecologically relevant trait.

The big picture that emerges here is that *Wolbachia* is extremely versatile in the sense that their effects on insect hosts can be extremely varied, depending on the insect species, the environment or even biotic conditions, one strain being able to switch from beneficial to deleterious over time, which makes any generalization impossible.

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CHAPTER 1

Influence of the symbiont
Wolbachia on
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Delia radicum

Chapter 1

Influence of the symbiont *Wolbachia* on the cabbage root fly *Delia radicum*

Context

Wolbachia is an intracellular bacteria, and probably the most abundant endosymbiont among insects, infecting more than half of all species in this group. In the earlier works following its discovery, *Wolbachia* was only considered as a reproductive parasite. Indeed, because this cytoplasmic symbiont is only transmitted by females (like mitochondria), the symbiont has evolved intriguing ways to interfere with key reproductive processes of its host, to enhance the proportion of infected females. However, recent years have witnessed the rapid accumulation of evidence that some *Wolbachia* strains can also benefit their hosts (without any reproductive manipulation: "stand-alone benefits") while other strains are clearly pathogenic and severely shorten the longevity of their host. The big picture that emerges is that *Wolbachia* is extremely versatile and that its effects on insect hosts can be extremely varied, depending on the insect species, the environment or even biotic conditions, one strain being able to switch from beneficial to deleterious over time, which makes any generalization impossible.

Approach

In this study, we investigated to what extent *Wolbachia* influenced several life history traits of the cabbage root fly (*Delia radicum*), a major pest of Brassicaceous crops in Brittany. From a large laboratory-reared fly stock, which was polymorphic for the infection, we created two separate infected and uninfected sub-stocks by pooling isofemale lines where the founder female had been PCR-typed for *Wolbachia* infection. This way, we obtained two strains bearing the same general genetic background but a different infection status: a 100% *Wolbachia*-infected stock (W+) and a *Wolbachia*-free stock (W-), so that we could assume that any observed differences between stocks would be due only to the infection status.

We then measured 10 life history traits and compared the results from the two strains: (i) probability to oviposit, (ii) number of eggs laid in 3 days (proxy of fecundity), (iii) probability of survival after ovipositing, (iv) egg hatch rate, (v) egg hatch time, (vi) emergence rate (probability to develop from egg to adult), (vii) egg-to-adult development time, (viii) sex-ratio (expressed as percentage of females), (ix) survival without food, and (x) adult size.

Apart from phenotypic traits, we also measured maternal transmission efficiency in the W+ strain, because the higher its value, the less important it is for the symbiont to increase its host's fitness to be maintained in the populations.

Results

Our results showed that the transmission of *Wolbachia* infection was 100% efficient in *D. radicum* and that the symbiont had various effects on LHTs, none being spectacular. Indeed, the symbiont had small negative, neutral or positive effects on its host, depending on the life history trait we measured, all of those effects probably compensating each other.

On the one hand, bearing *Wolbachia* had negative effects on starved *D. radicum* females when they laid eggs, since they suffered a 20% higher mortality during a 3-day oviposition period compared to uninfected females. However, the symbiont infection did not reduce viability in starving females which were not given the opportunity to lay eggs, suggesting that the energetic cost of the infection was only revealed in extreme conditions where unfed females had to exhaust their body reserves to produce eggs. Other negative effects of *Wolbachia* infection were a 10% lower hatch rate and a 1.5% longer development time from egg to adult. On the other hand, *Wolbachia* improved larvo-nymphal viability enough so that infected eggs, despite their reduced hatch rate, yielded as many adults as uninfected ones.

Finally, other traits did not seem to be influenced at all by the symbiont such as the egg laying probability, fecundity, egg hatch time, emergence rate, survival without food or adult size.

These moderate and compensating effects suggest at first sight that *Wolbachia* infection might be nearly benign in this host and might only drift slowly. This could explain why the infection rate has been stable in our laboratory (approximately 50% individuals infected) for many generations.

Limits

The study only focuses on one population from Brittany, and our experiments were made in the laboratory only, when we know that conditions in the field can be very different. In realistic and more stressful conditions in the field, it is possible that carrying *Wolbachia* comes with a cost for *D. radicum*, as we saw that infected females survival was reduced by 20% in extreme conditions. Therefore, we must interpret all these results with caution and cannot generalize them even if this study still gives us some good hints on what *Wolbachia* is able to influence in this species.

Perspectives

First, as vertical transmission of *Wolbachia* tends to be better in laboratory stocks than in the field, it would be necessary to check its efficiency in nature, using field-collected pupae of *D. radicum*. Also, as we know that the effects of *Wolbachia* can be variable across a range of environments, a next step would be to experiment the effects of the symbiont under conditions of abiotic/biotic stress. Finally, because of our lab-rearing stock observations, we hypothesized in our study that the *Wolbachia* variant studied here induced no or very little cytoplasmic incompatibility at most. A specific crossing experiment between infected and uninfected individuals would be necessary to confirm presence or absence of CI, because a weak CI can easily escape attention.

Journal of Invertebrate Pathology 158 (2018) 24–31

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Influence of the symbiont *Wolbachia* on life history traits of the cabbage root fly (*Delia radicum*)

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ARTICLE INFO

Keywords:
Delia radicum
Wolbachia
Symbiont
Endocellular bacteria
Life history traits
Maternal transmission

ABSTRACT

Wolbachia is an endocellular bacteria infecting arthropods and nematodes and is only transmitted vertically by females via the cytoplasm of the egg. It is often a manipulator of host reproduction, causing cytoplasmic incompatibility, thelytokous parthenogenesis, feminization or male killing, which all increase the proportion of infected females in the population. However, *Wolbachia* can modify life history traits of the host without causing the above phenotypes and each species illustrates the variability of relationships between this remarkably versatile symbiont and its many hosts. We have measured maternal transmission and the impact of a natural *Wolbachia* infection in the cabbage root fly *Delia radicum*, a major agricultural pest. We used a population that is polymorphic for the infection to ensure similar genetic and microbiome backgrounds between groups. Maternal transmission of the infection was 100% in our sample. We found no evidence of cytoplasmic incompatibility, thelytokous parthenogenesis, feminization nor male killing. *Wolbachia* infection significantly reduced hatch rate in infected eggs (by 10%) but improved larvo-nymphal viability sufficiently so that infected eggs nevertheless yielded as many adults as uninfected ones, albeit with a 1.5% longer total development time. Starved and infected ovipositing females suffered significantly reduced viability (20% higher mortality during a 3-day oviposition period) than uninfected females, but mortality was not higher in starved virgin females nor in starved males, suggesting that the energetic cost of the infection is only revealed in extreme conditions. *Wolbachia* had no effect on egg hatch time or offspring size. The apparently 100% vertical transmission and the significant but mutually compensating effects found suggest that infection might be nearly benign in this host and might only drift slowly, which would explain why the infection rate has been stable in our laboratory (approximately 50% individuals infected) for at least 30 generations.

1. Introduction

The term “symbiosis” in its most general – and original – sense is defined as the intimate “living together” of dissimilar organisms (De Bary, 1879). All insects, including pest species, are colonized by microorganisms, variously located in the gut and within insect tissues, the latter being called endosymbionts (Arora and Douglas, 2017). Bacterial endosymbionts are tremendously abundant among invertebrates, particularly among arthropods (Zchori-Fein and Bourtzis, 2011), having intimate and often obligatory associations with their host species (Werren and O’Neill, 1997), and functioning as ‘hidden players’ in many ecological and evolutionary processes (Frago et al., 2012). It is now widely recognized that the union of symbiont and host provides opportunities for the evolution of novel phenotypes by combining genomes with different biochemical capabilities (Werren and O’Neill, 1997).

Effects of symbionts on their hosts are diverse (Engel and Moran, 2013; Douglas, 2015; Graf, 2016) and can be beneficial or detrimental (Oliver et al., 2010), but assigning a symbiotic association to one of these categories is often problematic. The associations between symbiont and host are complex and can shift between different states over time, and also depend upon the phenotypes considered (Clay, 1988; Saffo, 1992). For example, endosymbionts can increase the resistance of their hosts to parasitoids (Oliver et al., 2003; Oliver et al., 2005; Vorburger et al., 2009; Heyworth and Ferrari, 2015; Donald et al., 2016) or fungi (Scarborough et al., 2005; Łukasik et al., 2013; Heyworth and Ferrari, 2015). They can also influence host fecundity, body mass (Castañeda et al., 2010; Vorburger et al., 2013), longevity (Fukatsu et al., 2001; Polin et al., 2014), body color (Tsuchida et al., 2010), growth and/or reproduction (Fukatsu et al., 2001) in a negative or positive way. Also, they can influence the host’s nutrition supply (Koga et al., 2003, 2007), resistance to heat shock (Chen et al., 2000;

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https://doi.org/10.1016/j.jip.2018.09.002
Received 22 June 2018; Received in revised form 27 August 2018; Accepted 1 September 2018
Available online 05 September 2018
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Russell and Moran, 2006; Guay et al., 2009; Heyworth and Ferrari, 2015) or even behavioral traits such as defensive behavior (Polin et al., 2014).

The cabbage root fly, *Delia radicum,* is a major pest of brassicaceous crops (e.g. rape, cabbage, turnip) where its belowground larvae feed on host plant roots (Griffiths, 1986). The microbiome was recently described (Bili et al., 2016) and revealed the presence of *Wolbachia sp.*, an endocellular symbiont that is vertically transmitted by females via the cytoplasm of the egg (Werren, 1997).

Wolbachia is the most abundant endosymbiotic bacteria among arthropods, infecting more than half of all species in this group (Martinez et al., 2016). Indeed, it is probably the most abundant intracellular symbiont on earth (Hilgenboecker et al., 2008; Zug and Hammerstein, 2012; Zug and Hammerstein, 2015). Many *Wolbachia* variants increase their prevalence in host populations by manipulating host reproductive systems (O'Neill et al., 1997), which enhances the proportion of infected females and thus benefits the maternally inherited symbiont (Werren et al., 2008). Mechanisms include the induction of feminization (FI) which results in genetic males that develop as females; thelytokous parthenogenesis induction (PI), which results in diploid females developing from unfertilized eggs; male killing (MK), which eliminates infected male embryos to the advantage of their infected female siblings; and cytoplasmic incompatibility (CI) which kills the embryos of uninfected females when they mate with infected males, while infected females are compatible with both infected and uninfected males (Werren et al., 2008).

Together, FI, PI, MK and CI represent alternative strategies to mutualism, which allow *Wolbachia* to persist in host populations. Because reproductive manipulations provide a strong fitness advantage to infected cytoplasmic lines, *Wolbachia* may not be disadvantaged by decreased fitness of infected females due to impacts on other traits, despite being a vertically transmitted symbiont (Werren, 2011). For example, feminizing *Wolbachia* are widespread among woodlice (Isopod) hosts even though they impose a fitness cost on infected neofemales (i.e. feminized males) because uninfected genetic males prefer uninfected genetic females over infected neofemales. Neofemales have lower mating rates and receive less sperm as a result (Moreau et al., 2001). Likewise, male-killing *Wolbachia* can increase to high prevalence but they strongly reduce the fitness of infected females by killing their sons; from the *Wolbachia* perspective, only daughters are important because only they can transmit the symbiont (Jiggins et al., 2002). *Wolbachia* infection has also been found to reduce fecundity of female hosts (CI: Hoffmann et al., 1990; PI: Stouthamer and Luck, 1993; CI: Perrot-Minnot et al., 2002).

However, even for reproductive parasites, it is an advantage to enhance female host fitness, and Turelli (1994) showed by modelling that selection on CI-inducing *Wolbachia* would favor variants that increase the relative fecundity of infected females. Vavre et al. (1999) were the first to report increase in fecundity due to *Wolbachia* infection without any evident reproductive manipulation ('stand-alone benefit' infection) in the parasitoid wasp *Trichogramma bourarachae*. There has been a recent upsurge of reports on *Wolbachia*-associated fitness benefits, and the symbiont has been shown in the laboratory to play defensive roles for its insect hosts (Zug and Hammerstein, 2015; Martinez et al., 2016) or to help with nutritional uptake (Hosokawa et al., 2010). This has also been demonstrated in the field where *Wolbachia* increased lifetime reproductive success in the wasp *Anagrus sophiae* (Segoli et al., 2013). It can especially be assumed that any *Wolbachia* variant that does not manipulate reproduction in the classical sense (FI, PI, MK or CI) must show a positive impact on female host fitness to be maintained in the host population.

In this study, we have aimed to establish the phenotype of *Wolbachia* infection in *D. radicum,* taking advantage of a mass-bred fly stock that is naturally polymorphic for the infection. By random sampling followed by typing for the infection, two large groups with a similar genetic background and differing only in infection status were obtained without the use of antibiotic treatments (such treatments are ideally avoided because they can themselves have an impact on life history traits). We measured *D. radicum* female fecundity, probability of oviposition, probability of survival after oviposition in starving conditions, hatch rate and time of eggs, probability that an egg will to develop into an adult, total development time, sex ratio, adult size and survival without food in unmated offspring. We also measured maternal transmission efficiency because the higher its value, the less important it is for the symbiont to increase fitness in females. Our results indicate a potential of 100% transmission as well as relatively small positive and negative fitness effects on the host. We found no evidence of FI, PI, MK or CI, although a low level of CI might have escaped detection in our protocol, which was not specifically designed to study this effect.

2. Material and methods

2.1. Insect culture

The *Delia radicum* strain studied here was originally reared from 4000 pupae collected in 2014 in experimental broccoli fields at Le Rheu (Brittany, France, 48°07′16″N, 1°47′41″O). After emergence, the flies were mass bred in the laboratory in a climate-controlled room (21 ± 1 °C, 60 ± 10% RH, L16:D8) on swede roots (*Brassica napus* subsp. *rapifera*) following a method derived from van Keymeulen et al. (1981). During preliminary tests, our lab *D. radicum* cultures were repeatedly sampled over 3 years (approximately 30 generations) and their infection status was verified by polymerase chain reaction (PCR) as described below. These tests showed that the flies are infected by the intracellular bacteria *Wolbachia*, prevalence being 50 ± 5% in both sexes.

No specific permissions were required for this experiment which did not involve endangered or protected species.

2.2. Creation of infected and uninfected lines with the same genetic background

The *D. radicum* stock was used to create two separate sub-stocks bearing the same general genetic background but a different infection status: a 100% *Wolbachia*-infected stock (W+) and a *Wolbachia*-free stock (W-), so that we could assume that any observed differences between stocks would be due only to the infection status. This assumption also supposed random mating in our rearing, which was likely since assortative mating according to *Wolbachia* infection status has been described only rarely (e.g. Vala et al., 2004; Champion de Crespigny et al., 2005).

All experiments were carried out in a climate-controlled room (20 ± 1 °C, 60 ± 10% RH, L16:D8). In our lab culture, 320 females and 200 males aged 1–5 days were sampled randomly in several insect rearing cages containing more than 5000 flies overall. This generation zero (G_0) was placed in a new rearing cage (Bug Dorm-4 Insect Rearing Cage, 47.5 × 47.5 × 47.5 cm) with wet cotton as a water source and a 1:1:1 mix of sugar, milk powder and dietary yeast *ad libitum* as food. Ten days later, the G_0 males were discarded and the 295 surviving G_0 females were placed individually in transparent plastic Solo Cups (Urbana, IL; diameter 4.2 cm, height 3.2 cm) where they could lay their eggs on a small piece of swede root (2 × 2 cm) resting on damp filter paper. During oviposition, females were not provided with food to increase the chances to detect a physiological cost of the infection. Three days later, eggs were collected. Of 295 females, only 78 (26.4%) laid eggs during that period of starvation. Eggs from each female were counted and placed on whole turnip roots (*Brassica rapa* subsp. *rapa*) according to the insect culture method mentioned in Section 2.1. (van Keymeulen et al., 1981). We used one turnip per female to establish isofemale lines.

2.3. Molecular analysis for verification of Wolbachia infection status

After collecting the eggs, G_0 females were euthanized by deep-freezing at −20 °C, stored individually in 0.5 ml Eppendorf tubes in 96° ethanol and tested to check their *Wolbachia* infection status (W+ or W−). For each individual, DNA was extracted following the salting-out method described in Sunnucks and Hales (1996).

Wolbachia status for all individuals was assessed by PCR using primers FbpA_F1 (forward) 5′-GCTGCTCCRCTTGGYWTGAT-3′ and FbpA_R1 (reverse) 5′-CCRCCAGARAAAYYACT ATTC-3′ (Simões et al., 2011).

The PCR reaction mixture (25 µL) consisted of 0.1 µL of DNA polymerase (3.5 U/µL), 0.5 µL of each primer (10 µM), 0.5 µL of dNTPs (10 mM each), 1.5 µL of $MgCl_2$, 5 µL of 5X PCR buffer and 1.2 µL of extracted DNA completed by 15.7 µL of ultrapure water. The PCR parameters were 94 °C for 2 min; 36 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min; and a final step at 72 °C for 10 min.

2.4. Maternal transmission rate of Wolbachia

All G_1 individuals (N = 467) from W+ females were tested by PCR as described above to check their *Wolbachia*-infection status (W + or W−) in order to establish the *Wolbachia* transmission rate in this strain. In case some infected G_0 females would have escaped detection because of a weak infection, 1–15 offspring(s) per G_0 W- females (N = 240) were also tested to search for false G_0 negatives (*Wolbachia* can only be transmitted by females via the cytoplasm of the egg so the presence of even a single infected offspring in a brood proves that the mother was infected).

Prior to PCR testing, developmental life-history traits and survival without food were measured on all these 707 individuals (see below).

2.5. Reproductive life-history traits on G_0 mothers

The probability to oviposit (marked 1 or 0 if the female oviposited or not, respectively), the number of eggs laid in 3 days (proxy of fecundity) and the probability of survival after ovipositing (marked 1 or 0 if the female was alive or not, respectively, after the 3-day starving experiment) were compared between W+ and W− G_0 mothers. To increase our original sample size for these measurements, 242 additional females sampled randomly in our lab rearing were individually isolated to oviposit according to the same protocol. Three days later, 142 of these females oviposited (58.7%). The eggs were counted, then discarded. These females were also tested to check *Wolbachia* infection status (W+ or W−).

2.6. Hatching rate for G_1 eggs

To measure egg hatching rate, pieces of swede roots (5 × 5 cm - resting on damp filter paper in a Petri dish) were placed in 4 cages (two cages of W+ flies and two of W− flies) each containing 150–200 adults. After 3 days, several thousands of eggs were collected for each treatment (W+ and W−). Eggs were sampled randomly from each of the Petri dishes and placed on wet filter paper in small closed cups, with 10 eggs per cup. In total, 151 cups of eggs from W- females and 150 cups of eggs from W+ females were checked daily for newly hatched larvae. Newly-hatched larvae were removed daily until no hatch occurred for an additional 7 days.

2.7. Developmental traits and survival without food

After *Wolbachia*-infection status of the mothers were known, each pot containing the infested turnip was tentatively identified as a W+ or W− isofemale line. The relative fitness of G_1 offspring from the W+ and W− lines was assessed by comparing several life-history traits: (i) emergence rate (probability to develop from egg to adult; i.e. the number of adults obtained/number of eggs laid), (ii) total development time (from oviposition to imago emergence), (iii) sex-ratio (expressed as percent females), (iv) survival without food in G_1 individuals; the first 1–15 emerging G_1 offspring from each G_0 female were placed individually in cotton-plugged 9.5 mm × 2 mm Plexiglas tubes when they were less than 24 h old. Each tube also contained a wet cotton ball as a water source, but no food. A piece of loose hydrophobic cotton was used to plug the tubes. These individuals were then monitored daily at the same time and survival was therefore measured in days, (v) adult size (dead flies from the survival experiment were measured under a dissecting microscope fitted with a Euromex CMEX5 camera using the Image Focus ® software. As a proxy of body size, we measured the tibia on the right third leg of each fly, with a ± 0.1 mm accuracy).

2.8. Statistical analysis

The efficiency of *Wolbachia* transmission from a G_0 mother to its G_1 offspring was estimated using a 95%CI based on an exact binomial test.

The probability for *D. radicum* females to oviposit depending on their infection status was analyzed using a generalized linear model (GLM) with a binomial error family and a logit link function. Because the data were collected during several experiments, the experiment was also added as a fixed effect:

$$y \sim infection\ status * experiment$$

The same model was used to test the fecundity of females, however, a quasipoisson error family was used, with a log link function.

A GLM with a quasibinomial error family and a logit link function was used to test for probability of females to survive after oviposition, and the number of eggs laid per female was also added as a fixed effect:

$$y \sim infection\ status * experiment + number\ of\ eggs$$

The hatching probability and hatching time analysis was analyzed using generalized linear mixed models (GLMMs) with, respectively, a binomial error family (logit link function) and a poisson error family (log link function). Because the eggs were split into 301 groups of 10 eggs, the group was used as a random factor in the statistical modelling:

$$y \sim infection\ status * experiment + (1|group)$$

The emergence rate (probability to develop from egg until adult) and sex ratio were analyzed using generalized linear models (GLMs) with a quasibinomial error family and a logit link function. The experiment and the number of eggs laid per female were also added in the model:

$$y \sim infection\ status * experiment + number\ of\ eggs$$

Development time, adult size and survival without food were analyzed using linear mixed models (GLMMs) with a gamma error family and an inverse link function. When analyzing development time, sex was added as a fixed effect and the number of eggs laid by the mother as a covariate. Since several offspring came from the same female, the mother was considered as a random factor:

$$y \sim infection\ status * sex + number\ of\ eggs + (1|mother)$$

To test for adult size, development time was added as a fixed factor:

$$y \sim infection\ status * sex + number\ of\ eggs + development\ time + (1|mother)$$

Survival without food was tested by including the size of the individual in the model:

$$y \sim infection\ status * sex + number\ of\ eggs + adult\ size + (1|mother)$$

After statistical modelling, pairwise comparisons between infected and uninfected insects were performed using least-squares means. The mean values obtained and their associated standard errors were used to make the graphs.

All statistics were performed using the R.3.0.2 software (R Core Team, 2017) with packages car (Fox and Weisberg, 2011) for GLMs, lme4 (Bates et al., 2015) for GLMMs and lsmeans (Lenth, 2016) for pairwise comparisons.

3. Results

3.1. Transmission rate

A total of 611 *D. radicum* originating from 68 different mothers were tested for *Wolbachia* via PCR. All 219 offspring of the 29 W− mothers were *Wolbachia*-free and all 392 offspring of the 39 W+ females were tested positive for *Wolbachia*. Maternal transmission rate of *Wolbachia* was possibly as high as 100% in our experimental conditions (lower bound of $_{95\%}$CI: 98.6%).

3.2. Reproductive traits

Wolbachia infection significantly reduced the survival of starved fecund females and egg viability: infected fecund females were 20% less likely to survive during the 3 days of the experiment than uninfected fecund females (Fig. 1a; LR Chisq = 6.55, 1 df, $P = 0.01$) and eggs laid by infected females had a 10% lower hatch rate (Fig. 1b; Chi Sq = 23.2, 1 df, $P < 0.001$). Other traits were not modified: the probability to oviposit (uninfected: 80.4 ± 3.7%, infected : 83.3 ± 3%, LR Chisq = 0.012, 1 df, $P = 0.91$), the number of eggs oviposited by fecund females during a 3-day period (uninfected: 29.1 ± 2 eggs, infected: 26.8 ± 1.7 eggs, Sum Sq = 4, 1 df, $P = 0.59$) and hatching time were not significantly different between uninfected and infected flies (uninfected: 6.1 ± 0.1 days, infected: 6.2 ± 0.1 days, Chisq = 0.07, 1 df, $P = 0.79$).

3.3. Developmental traits

Despite a significantly lower hatch rate (–10%, see above), the probability of infected individuals to develop from egg to adult was at least as good as that of uninfected individuals (Fig. 2a; LR Chisq = 1.56, 1 df, $P = 0.21$), which indicates a better larvo-nymphal survival in infected individuals. Total development time was significantly but negligibly slower in infected individuals (by 1.5%, i.e. half a day during a month of development, Fig. 2b; LR Chisq = 56.02, 1 df, $P < 0.001$).

Sex ratio (expressed as percent female offspring) was nearly identical between uninfected and infected strains (SR_{W-} = 49.1 ± 2.8% and SR_{W+} = 48.7 ± 1.7%, LR Chisq = 0.0001, 1 df, $P = 0.99$), and was not significantly different from a 50:50 sex ratio in both strains (Chisq = 0.43, 1 df, $P = 0.51$ for W− and Chisq = 0.74, 1 df, $P = 0.39$ for W+).

3.4. Size and survival without food

Infection by *Wolbachia* did not influence adult size as estimated via the tibia of the third leg (uninfected: 1.89 ± 0.03 mm, infected: 1.86 ± 0.02 mm, Chisq = 0.47, 1 df, $P = 0.44$). Although *Wolbachia* infection significantly reduced survival by 20% in the fecundity experiment, it did not significantly reduce survival in starved but unmated individuals (males or females) that were not provided with an oviposition site (uninfected: 6.97 ± 0.11 days, infected: 6.62 ± 0.07 days, Chisq = 1.23, 1df, $P = 0.27$).

4. Discussion

4.1. Transmission rate

4.1.1. A perfect transmission rate in the lab

For vertically transmitted endosymbionts like *Wolbachia*, vertical transmission efficiency is obviously one of the most important factors for successful maintenance in the host populations, because poor vertical transmission would require spectacular positive or manipulative effects in the host to compensate losses at each generation (Hoffmann et al., 1990, 1998; Turelli and Hoffmann, 1995; Werren, 1997). In our study, all 392 offspring of the 39 infected mothers were infected, which suggests that vertical transmission might be 100%, or nearly so, in this species. Such a result is not an isolated case: Narita et al. (2009) found a 100% vertical transmission rate of *Wolbachia* in the butterfly *Colias erate poliographus,* and Walker et al. (2011) observed 100% maternal transmission of the *wMel* strain in the mosquito *Aedes aegypti*. However, 100% transmission of *Wolbachia* is more the exception than the rule, and depending on strains and host species, uninfected individuals are typically produced in low to significant proportions (1% in McMeniman et al., 2009 , 4–10% in Poinsot et al., 2000, 10–30% in Turelli and Hoffmann, 1995, 15–66% in Duplouy et al., 2015), and sometimes the infection can be lost altogether from small lab stocks because of drift or sub-par breeding conditions for the host (Merçot and Poinsot 1998). Nevertheless, Hoffmann et al. (1990) showed that maternal transmission of *Wolbachia* tends to be better in laboratory stocks than in the field, probably because hosts are usually maintained in ideal

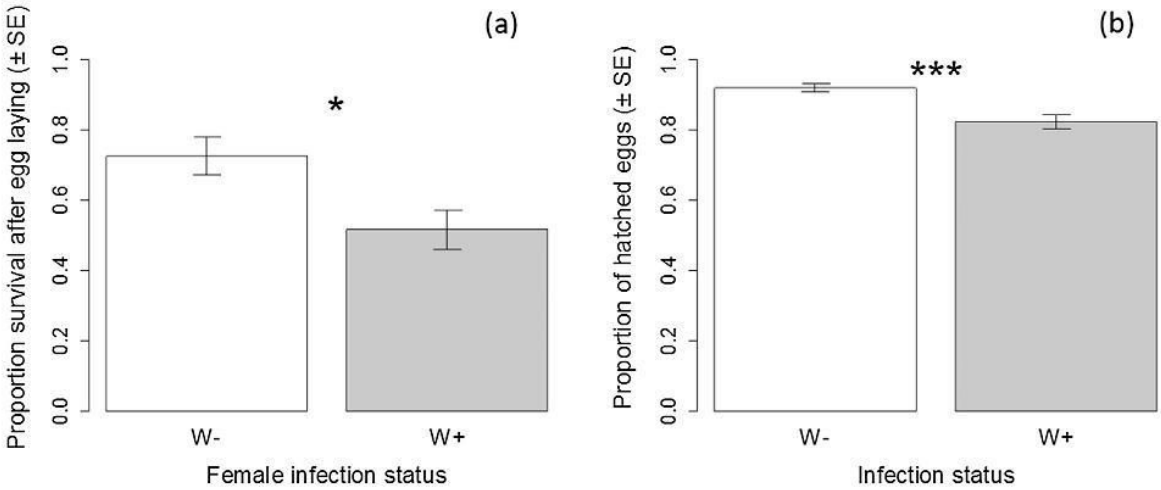


Fig. 1. Effect of the *Wolbachia* infection status on (a) the probability for *Delia radicum* females to survive after egg laying (N_{W-} = 75 and N_{W+} = 82) and (b) the probability for an egg to hatch depending on its infection status (N_{W-} = 1510 and N_{W+} = 1500). Individuals free from *Wolbachia* in white (W-) or infected with *Wolbachia* in grey. Bars: standard error. *: $P < 0.05$, ***: $P < 0.001$.

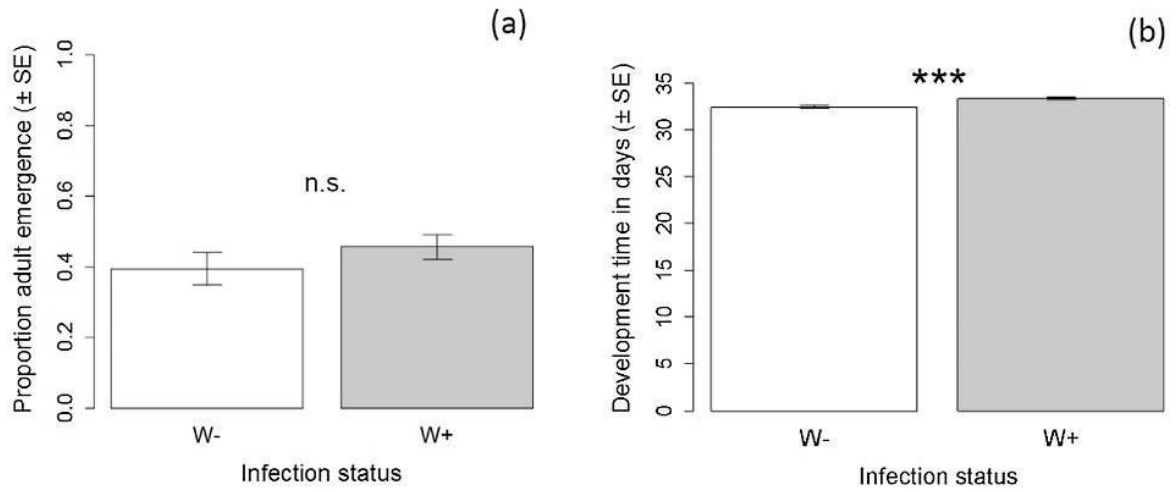


Fig. 2. Effect of the *Wolbachia* infection status on (a) the probability of emergence (N_{W-} = 48 and N_{W+} = 84) and (b) development time from egg to adult (N_{W-} = 156 and N_{W+} = 535. Individuals free from *Wolbachia* in white (W−) or infected with *Wolbachia* in grey. Bars: standard error. n.s.: not significant. ***: $P < 0.001$.

temperature and food conditions that also benefit their symbionts. It is, therefore, possible that the 100% transmission we observed overestimates the situation in the field. Moreover, an absolutely perfect transmission rate is also difficult to imagine as flies could possibly feed on substrates that might contain natural antibiotics and *Wolbachia* is sensitive to increases of temperatures (Stevens, 1989). In addition, flies can be subjected to thermal shocks in the wild that might eliminate their symbionts. This question should be investigated using field-collected pupae of *D. radicum*. Finally, efficient vertical transmission of the symbiont suggests a long history of association between this *Wolbachia* and *D. radicum*. This would be consistent with the relatively modest effects of the infection we found on life history traits of its host: unlike old associations, recent infections are more likely to result in antagonist phenotypes and imperfect transmission, evidence of lack of coadaptation between the two partners (Turelli and Hoffmann, 1991; Turelli, 1994; Poinsot and Merçot, 1997, but see Walker et al. 2011).

4.1.2. No apparent cytoplasmic incompatibility in D. Radicum

Wolbachia often induces cytoplasmic incompatibility (CI) in insects; it is, indeed, the most common type of host manipulation caused by this symbiont (Bourtzis and Miller, 2003). Because *Wolbachia* is only transmitted through females, cytoplasmic incompatibility is expected when an infected male mates with an uninfected female, resulting in embryonic mortality of uninfected eggs, while infected females are compatible with all males (Werren, 1997; Bourtzis and Miller, 2003; Poinsot et al., 2003). In our strain, the apparently 100% vertical transmission of *Wolbachia* means that any significant cytoplasmic incompatibility should lead to a rapid spread of the symbiont in the host population, as has been observed in other studies, including in nature where transmission is not perfect (Turelli and Hoffmann, 1991). However, in our original *D. radicum* laboratory stock, which is polymorphic for the infection, the prevalence of *Wolbachia* has been stable for 3 years in the lab (over 30 generations), being about 50 ± 5% in both sexes. In this population, it is thus most likely that the *Wolbachia* variant studied here induces no or very little cytoplasmic incompatibility at most. A specific crossing experiment would be necessary to confirm presence or absence of CI, because a weak CI can easily escape attention; 80 years of intense genetic studies involving innumerable crossing experiments in *Drosophila melanogaster* were conducted before CI was detected (Morgan 1910, Hoffmann, 1988).

4.2. Life history traits

4.2.1. Fecundity is not influenced by Wolbachia in the cabbage root fly

Vertically transmitted symbionts bear an especially strong selective pressure against reducing host fecundity, and they can improve their own fitness by increasing the reproductive output of their hosts (Turelli, 1994; Zug and Hammerstein, 2015). *Wolbachia* was historically interpreted to be a reproductive parasite (Werren, 2011), and was found in some cases to reduce host fecundity in the lab (Hoffmann et al., 1990; Stouthamer and Luck, 1993; Perrot-Minnot et al., 2002). However, other studies have revealed positive effects on host fecundity in various insect such as the drosophilids *Drosophila melanogaster* (Dobson et al., 2004; Fry et al., 2004) and *Drosophila simulans* (Weeks et al., 2007), the mosquitoes *Aedes albopictus* (Dobson et al., 2002) and *Culex pipiens* (Zélé et al 2017), and the parasitoid wasps *Nasonia vitripennis* (Stolk and Stouthamer, 1996) and *Trichogramma bourarachae* (Vavre et al., 1999). In the hymenopteran *Asobara tabida* and in the collembolan *Folsomia candida*, losing the infection even results in total female sterility (Dedeine et al., 2001; Pike and Kingcombe, 2009).

Therefore, the effects of *Wolbachia* on host fecundity can be positive or negative and are not based on phylogenetic relationships among the hosts. In the present study, *Wolbachia* appeared to be relatively benign in terms of host fecundity: it neither increased nor decreased the probability for *D. radicum* females to oviposit during a 3-day experiment, nor did infection modify the number of eggs laid by fecund females. Likewise, *Wolbachia* had no effect on fecundity in a study made on the wasp *N. vitripennis* using a similar protocol: the strains compared had the same genetic background and fecundity was measured for a short period of time (i.e. less than 6 h; Bordenstein and Werren, 2000). Although our study does not reflect the effect of *Wolbachia* on lifetime fecundity, the ovipositing females in our experiments were not fed so that the fecundity we measured represented stressful trophic conditions. It is therefore noteworthy that the unavoidable energetic cost of the infection did not reduce fecundity even in this very demanding situation (but see further for the effect on survival).

4.2.2. Wolbachia reduces egg viability, improves larvo-nymphal viability, and increases development time

In our *D. radicum* strain, eggs from infected mothers required the same hatch time as eggs from uninfected mothers, but hatching success was 10% lower, highly statistically significant ($P < 0.001$). Joshi et al. (2014) found a similar reduction (–15%) in the viability of infected eggs of the mosquito *Anopheles stephensi*. We suggest that either (i) infection

had a negative effect on the ovipositing females, which indirectly reduced the viability of their eggs (e.g. smaller eggs, poorer quality of egg yolk) or (ii) bacterial load in the egg itself had a negative effect on embryo development (e.g. toxicity, competition for key nutrients). Caragata et al. (2013) suggested, for example, that *Wolbachia* reduced the viability of the mosquito *A. aegypti* by competing with the embryo for amino acids.

To rule out the hypothesis that *Wolbachia* affected the capacity of females to produce viable eggs, it would be technically possible to measure or weigh the eggs to determine if eggs of infected females are indeed smaller. It would also be feasible to supplement females with key nutrients to determine whether infected eggs remain disadvantaged. Studies of direct trophic competition between *Wolbachia* and the embryo *in situ* would seem a much more challenging task.

4.2.3. Larvo-nymphal variability

Because the hatch rate of infected eggs was statistically significantly lower (10%) than those of the uninfected strain, we expected infected eggs to yield significantly less adults, which did not happen. In our sample, the proportion of emerging adults in the infected group was higher by 6% than in the uninfected group but the increase was not statistically significant. The capacity of infected larvae to produce as many adults as higher numbers of uninfected larvae in our experiment implies higher larvo-nymphal viability in infected individuals, and allows us to conclude that infected females produce as many offspring as uninfected females under our experimental conditions. We also found that development time was slightly slower in infected flies, but this effect, although statistically significant, may be negligible (a 1.5% increase). The symbiont thus seems to play both a negative role in eggs and a positive one in larvae, a trade-off that ensures that offspring productivity is not reduced. The mechanisms by which *Wolbachia* could enhance larva-to-adult survival in *D. radicum* are unknown. Hosokawa et al. (2010) and Nikoh et al. (2014) showed that in the bedbug, *Cimex lectularius, Wolbachia* supports host growth and survival via provisioning of specific vitamins. In *C. lectularius*, however, *Wolbachia* has become an obligatory symbiont, which is not the case in *D. radicum*.

For all flies, whether they were infected or not, the probability of emergence was rather low ($W- = 0.39 \pm 0.05$ and $W+ = 0.46 \pm 0.03$) but this was not unexpected because another study using the same experimental conditions (*Delia radicum* reared in a climate-controlled room at 21 ± 1 °C, 60 ± 10% RH, L16:D8, on swede roots) also showed low emergence rates on various plants (18%–37%) (Van Geem et al., 2015).

4.2.4. Wolbachia reduces survival but only in starving fecund females

In our study, *Wolbachia*-infected females had a 20% lower survival probability compared to uninfected females when they oviposited for 3 days without food, while infection did not significantly reduce the survival probability of unmated females (or males) in the same starving conditions but no possibility to oviposit. These results demonstrated that *Wolbachia* infection in *D. radicum* adds a physiological cost to the host, and that this cost is low enough to be undetectable even in drastic "simple" starving conditions but high enough to be revealed when the demands of oviposition are added to starving conditions. The infected fecund females showing reduced survival were mated and all produced offspring. Their survival could thus be affected by *Wolbachia* either by the cost of mating (see studies by Bilewicz, 1953; Chapman et al., 1995) or the production of eggs. Maynard-Smith (1958) showed that reproductive processes can shorten lifespan in the fruit fly *Drosophila subobscura*, so that female longevity can be improved by reducing the rate of egg-laying. At the physiological level, trade-offs between survival and reproduction can be caused by competitive allocation of limited resources into the production of eggs versus somatic maintenance and survival (Flatt, 2011).

We can exclude the hypothesis that *Wolbachia* could reduce female viability in *D. radicum* by increasing clutch size (draining the body's nutrient pool faster), because infection did not modify fecundity during the 3 days of our experiment. It is also unlikely that *Wolbachia*-infected females allocated more body reserves to each egg, thus reducing female viability. Indeed, although such a manipulation of the host would obviously be advantageous for the symbiont, it seems contradictory with our observation that infected eggs had a significantly lower hatch rate. A more likely explanation is that the baseline physiological cost of the symbiont was too high for a starving female using its last reserves to produce eggs. At this stage, we favor this hypothesis. If correct, the reduced survival we found might be unlikely in field conditions where *D. radicum* females typically do not lay eggs until death by starvation but alternate between field margins where they feed and host plants where they oviposit (Hawkes, 1972).

As previously stated, with the exception of ovipositing females, we did not find any effect of the symbiont on survival of our *D. radicum* strain even in starving conditions. Very few studies have focused on the effects of *Wolbachia* on survival. In the mosquito *A. albopictus, Wolbachia* infection shifts from parasitism to mutualism as a function of developmental conditions: infected females have reduced survival under highly competitive conditions but better survival than uninfected females when competitive pressures are low (Gavotte et al., 2010). In the related species *Aedes polynesiensis*, infected individuals lived longer than aposymbiotic ones, however the aposymbiotic strain used was created using antibiotics (tetracycline), which could have reduced survival (Brelsfoard and Dobson, 2011). Other studies found a positive effect of *Wolbachia* on survival or longevity (Fry et al., 2004; Alexandrov et al., 2007; Dong et al., 2007; Toivonen et al., 2007; Xue et al., 2012) but all of them also used antibiotics (tetracycline or/and rifampicin) to create aposymbiotic strains, which does not exclude a possible direct effect of the antibiotic, which can last for several generations (Ballard and Melvin, 2007). Using *D. radicum* from a strain that is naturally polymorphic for infection by *Wolbachia*, we showed that the symbiont does not represent a cost sufficient to alter viability even in starving conditions, unless the females are further draining their resources by laying eggs.

4.2.5. No effect of Wolbachia on sex ratio (no MK nor FI) and size

Wolbachia did not cause detectable sex-ratio distortion in favor of *D. radicum* females. *Wolbachia* is well-known to distort sex-ratio in favor of infected females in some species, and exhibits great versatility in strategies for achieving it by inducing either feminization, male killing, or thelytokous parthenogenesis (Werren et al., 2008). However, *Wolbachia*-induced SR distortion has rarely been shown in Dipterans (except in *Drosophila bifasciata* - Hurst et al., 2000 and *Drosophila innubila* - Unckless and Jaenike, 2012) so its absence here is not surprising.

We did not find a significant effect of *Wolbachia* on *D. radicum* adult size. Compared to fecundity or survival, the effect of *Wolbachia* on this trait has been little studied. Brelsfoard and Dobson (2011) found smaller wings in *Aedes polynesiensis* individuals cured from their *Wolbachia* infection by an antibiotic treatment (tetracycline) followed by three generations before the experiment, to allow the recovery of the gut microbiome. Likewise, Xue et al. (2012) found a smaller larger body size in the whitefly *Bemisia tabaci* cured from their *Wolbachia* by rifampicin, and suggested that *Wolbachia* may confer some nutritional benefits on the host. In our study, where a large cage population was sampled and groups were created based on *Wolbachia* infection status, we conclude that the symbiont did not have a detectable effect on sex ratio nor the size of *D. radicum* adults as estimated by tibia length. A similar result was found in *D. melanogaster* by Arbuthnott et al. (2016), where the authors studied body mass.

5. Conclusion

We found that the *Wolbachia* infection of *D. radicum* was 100% vertically transmitted, that it presents no evidence of the four "classical" reproductive manipulation phenotypes (feminization,

parthenogenesis, male-killing or cytoplasmic incompatibility), but that it shows moderate positive and negative effects on other traits (effect sizes in the 1–20% range). We observed in particular that *Wolbachia* reduced survival in starving ovipositing females, but not in starving unmated individuals. We also found that *Wolbachia* infection reduced hatch rate but improved larvo-nymphal survival. As a result, infected individuals produce as many adults as uninfected individuals, with a very slightly longer larvo-nymphal development time. Finally, we found no effect of infection on adult size. The partial picture that emerges at this stage of our investigation is that *Wolbachia* seems to behave in *D. radicum* as a relatively benign infection that drifts very slowly through the host population – in our lab, infection prevalence has remained stable at approximately 50% for three years. Our other preliminary field results (data not shown) suggest a low prevalence in any given population but ubiquity at a larger scale. The lack of any spectacular effect of *Wolbachia* on *D. radicum* also means that, at this early stage at least, *Wolbachia* does not seem a promising candidate to be used as a lever to control this important agricultural pest.

Acknowledgments

We thank Chrystelle Paty and Alexiane Kustre for helping with the numerous counts of eggs and measurements of fly legs. We are also grateful to Dr. Maxime Hervé and Pr. Yannick Outreman for their precious help with data analyzing. We express our gratitude to Dr. Hervé Merçot for helpful comments on the manuscript. Last but not least, we thank the reviewers for their helpful comments and suggestions, and the University of Rennes 1 that provided the funding and made this work possible.

Disclosure

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jip.2018.09.002.

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CHAPTER 2

Influence of gut microbiota
on the life history traits
of *Delia radicum*

Chapter 2

Direct and indirect effects of tetracycline on life history traits of the cabbage root fly (*Delia radicum*)

Context

All insects, including pest species, are colonized by microorganisms, either with bacteria, fungi, viruses or protozoa. Amongst these taxa, bacteria are probably the most prevalent and can be located in the gut, within insect tissues or even inside the cells, the latter being called endosymbionts. Many studies focused on the interaction that hosts can have with these bacterial endosymbionts because they are tremendously abundant among insects and are known to have important and various effects on their hosts' phenotypes. Indeed, they basically bring "ready-to-use" functions that can be beneficial or not for their hosts. Because they are heritable, some endosymbionts can become indispensable for survival, development or reproduction of their hosts, and thus influence their evolutionary trajectory. However, more and more studies have been focusing on gut microbiota lately because we are coming to understand that these "external" symbionts might be as important as intracellular bacteria in modulating host physiology, ecology, behavior, and evolution. Insects are particularly good models for studying gut microbiota because their bacterial diversity is relatively simple, with typically less than 30 taxa per host. To evaluate the effects of gut microbiota on life history traits, many studies used large-spectrum antibiotics to eliminate gut bacteria and then compared the aposymbiotic (= free from bacteria) strain to the control, untreated one. With this protocol, it is often assumed that the effects observed are due to the loss of symbionts only, and not a possible direct toxic effect of the antibiotic.

Approach

In this study, we have aimed at teasing out the possible direct effects of tetracycline on the host (chemical effect) from its indirect effects (due to the partial or total loss of gut microbiota).

The antibiotic we chose for this experiment was tetracycline as it is very commonly used to treat a variety of bacterial infections. It is already known that tetracycline treatment reduces mitochondrial efficiency and probably leads to decreased ATP production in *Drosophila simulans*, probably because of the "bacterial" origin of mitochondria. Obviously, this could have a direct influence on life history traits of the host, which may easily be confused with a "bacteria loss" effect. The fact that we used a *Wolbachia*-free line ensures that the possible effects observed would not be due to this endosymbiont but only to the modification of the gut. A protocol spanning three generations was established, which allowed us to discriminate the possible direct effect of tetracycline on the host (chemical effect) from its indirect effects (due to the partial or total loss of gut microbiota).

We then measured six classical life history traits: the probability for an egg to develop into an adult, total development time, sex ratio, adult size, survival without food in unmated individuals and egg load in offspring females (number of mature eggs in the ovaries after 10 days in presence of males).

Results

All life history traits but sex ratio were affected (mostly negatively) by the tetracycline treatment. Moreover, these effects could be detected up to two generations after treatment. Overall, emergence rate was lower, development time was longer, and survival was reduced, as well as egg load. The offspring of *parents* that were treated with tetracycline (with grandparents that had not been treated before), were the most severely impacted, with three out of five life history traits being significantly degraded (emergence rate, survival without food and egg load). The only trait that seemed to be improved by the tetracycline treatment was size, with one line treated yielding larger individuals when their *grandparents* had been treated with tetracycline but their parents had *not*. Our data suggest that the effect of microbiota complete or partial loss has a larger role than any toxic effect of tetracycline itself. This allowed us to conclude as well that the offspring gut microbiota is at least partially inherited from the mother. Overall, this study suggested a beneficial role of the "wild-type" gut microbiome in this species as the line with the best phenotype was, for each trait, the control one.

Limits

At the time of the writing, we do not know how the treatment really impacted the gut microbiota. We do not assume however that all bacteria were completely removed from the gut of treated individuals (parents and grandparents) as it is known that some bacteria taxa usually living in the gut are tetracycline-resistant. We hypothesize here that instead of becoming aposymbiotic, the lines treated had their microbiota greatly modified with tetracycline-insensitive bacteria multiplying across generations to occupy the niches liberated by the treatment, and mingling with bacteria contaminating the gut during the (untreated) larval stages. The absence of sequencing results prevents a solid conclusion on this point as we do not have a precise characterization of the gut microbiota of treated and untreated individuals. For this reason, the discussion of the following paper remains speculative.

Another limit of this study is that we do not have any measurements of life history traits for generation 0 (grandparents) and 1 (parents); the data we collected is for generation 2 only (offspring). Having data on the phenotypes of parents and grandparents would have been very helpful for more specific conclusions. This is because, originally, this protocol was designed to study *Wolbachia*, and not the gut microbiota. Indeed, this was the very first experiment we did during this thesis. At that time, we thought our lab strain was still infected with *Wolbachia* as had been shown before, and our purpose was to create a strain free from the endosymbiont by using tetracycline. To do so, we decided to do this protocol during three generations to create four lines: a control one that would not get any treatment, a line where only parents would be treated to account for the tetracycline effect, a line where only grandparents would be treated to account for the *Wolbachia* effect (as the parents would have been free from *Wolbachia* but would have recovered a gut microbiota) and a line where parents *and* grandparents would have been treated to account for the additive effect of *Wolbachia* loss and tetracycline treatment. After eight months of experiments, we discovered that unfortunately, the lab strain we had used was actually free from *Wolbachia*. Because it was a lot of experimental work, and still an interesting experiment “gutwise”, we decided to adapt it to the study of gut microbiota. This is why the protocol we used here is not perfectly adapted, but still good enough to give us some clues on what experiments should be done next to complete this work.

Perspectives

To complete this work, we would need to know the richness and diversity of gut microbiota in our four kinds of treatments. By using molecular techniques, we could identify the predominant bacterial taxa in each line and identify the contribution of a specific bacteria to a specific phenotype. As we kept all the individuals we measured in this experiment, individually in 96° alcohol, we decided to sequence them. After extracting their DNA, we ran PCR on each of the flies to check for bacterial DNA presence and sent them to sequencing. Unfortunately, we will not get these samples back on time for this thesis manuscript, but a paper will be made as soon as we get them, as these data should nicely complete the work that has been made on life history traits of *D. radicum*.

In preparation for Journal of Insect Physiology

Depleting adult gut microbiota reduces the fitness of offspring in the cabbage root fly (*Delia radicum*)

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Abstract: Many studies on insects evaluate the effects of bacterial symbionts by eliminating them and comparing the performance of the resulting symbiont-poor host with that of untreated individuals. The most common method used to suppress bacteria is treating hosts with large-spectrum antibiotics, and it is implicitly assumed that tetracycline treatment has no other effect than removing bacteria. However, this is not always the case and effects attributed to the loss of gut microbiota might be in fact caused by a direct antibiotic toxicity toward the host. In this study, the effects of tetracycline were evaluated with a protocol spanning three generations, which allowed to discriminate the possible direct effect of tetracycline on the host (chemical effect) from its indirect effects (due to the partial or total loss of gut microbiota). Here, antibiotic treatment of *Delia radicum* adults led to multiple and mostly negative effects on life history traits of their offspring, that could be detected up to two generations after treatment. The offspring of parents that were treated with tetracycline (grandparents being untreated), were the most severely impacted, with three out of five life history traits being degraded (emergence rate, survival without food and egg load). Data also suggested that the indirect effect of gut microbiota perturbation had a larger role than any putative toxic effect of tetracycline itself, and also that this microbiota was at least partially inherited

maternally. A total destruction of the microbiota post treatment was not assumed here. Instead, a large modification of gut microbiota was hypothesized, with some tetracycline-insensitive bacteria taxa surviving tetracycline and multiplying to occupy the niches liberated by the treatment. Overall, this study suggested a beneficial role of the "wild-type" gut microbiota in this species.

Keywords: bacterial symbiont, gut microbiota, antibiotic, tetracycline, insect, life history traits, *Delia radicum*, cabbage root fly

INTRODUCTION

All insects are involved in numerous symbiosis, either with bacteria, fungi, viruses or protozoa (Richards & Brooks, 1958; Douglas, 1989). Amongst these taxa, bacteria are probably the most prevalent in terms of frequency and diversity (Pace, 1997). Insects have very diverse interactions with the bacteria they harbor but contrary to early conceptions, these microorganisms are not always pathogenic and often beneficial or even required by the insect host (Douglas, 2015; Raymann & Moran, 2018). For this reason, they are now neutrally referred to as "symbionts".

Insects can harbor bacterial symbionts basically anywhere. When bacteria live on the surface of their hosts - including internal surfaces such as the lining of the digestive tube and the ducts of glands - the term "ectosymbiosis" is used and such symbionts are always extracellular. On the contrary, "endosymbiosis" is used when the host harbors the bacteria within (intracellular) or between its own cells (extracellular). Intracellular endosymbionts thus live directly in the cytoplasm of their host's cells ("endocytobiosis") (Bull & Fogarty, 1996; Dubilier *et al.,* 2008; Goffredi, 2010).

Intracellular symbionts are widespread among insects and have been extensively studied because they are known to have important and various effects on their hosts' phenotypes. Indeed, they basically bring "ready-to-use" functions that can be beneficial or not for their hosts, and influence their evolutionary trajectory (Oliver *et al.*, 2010). Moreover, because they are heritable, some symbionts can become indispensable for survival, development or reproduction of their hosts (Moran, 2006; Guo *et al.*, 2017). For example, the obligate aphid symbiont *Buchnera aphidicola* is vital for its hosts as it provides them with essential amino acids they cannot obtain themselves from plant sap (Douglas, 1998; Shigenobu *et al.*, 2000; Baumann, 2005).

Increasingly, we are coming to understand that (extracellular) gut microbiota might be as important as intracellular bacteria in modulating host physiology, ecology, behavior, and evolution. Gut bacteria are now very well-known to upgrade nutrient-poor diets (Flint, 2012; Wong *et al.*, 2014), aid digestion of recalcitrant food components (Brune, 2014), protect from pathogens (Dillon *et al.*, 2005), detoxify plant compounds (Després, 2007), influence tissue development (Shin *et al.*, 2011) or circadian regulation (Leone, 2015), contribute to inter and intraspecific communication, influence mating and

reproductive systems (Archie & Tung, 2015; Morimoto *et al.*, 2017; Walsh *et al.*, 2017), or even drive speciation (Brucker & Bordenstein, 2013).

Insects are particularly good models for studying gut microbiota because their bacterial diversity is often relatively simple, with typically less than 30 taxa per host (Dillon & Dillon, 2004), as compared to over 1000 taxa per host in mammals (Dethlefsen *et al.*, 2007). Accordingly, the diversity of gut microbiota has been extensively studied recently, especially in some groups of economically important insects such as aphids (Douglas, 1998; Shigenobu *et al.*, 2000), termites (Nakashima *et al.*, 2002) and honey bees (Raymann & Moran, 2018).

The most common method to evaluate the effect of the microbiota is to reduce or eliminate it, and compare the performance of the resulting symbiont-poor host with that of untreated individuals. However, despite the explosion of interest in the insect gut microbiota in the past few years, there is still little consensus regarding the most effective method for eliminating it (Heys *et al.*, 2018).

In Dipteran species, two methods are commonly used to alter gut bacterial communities: antibiotic treatment (via the food or water) or removing the chorion from eggs. This second method is used because eggs are coated with highly diverse bacteria transmitted largely from fecal deposits from the mother during oviposition (Wong *et al.*, 2011). When larvae emerge from the egg, they ingest part of the chorion and the bacteria coating it, forming the basis of their microbial community (Bakula, 1967).

Both the use of antibiotics and dechorionation of the egg are widely applied, but also widely criticized due to the diverse adverse effects they could have on insect life history traits. Despite critics, and because it is a much simpler method, recent publications have favored the use of antibiotics, and particularly tetracycline, rifampicin, ampicillin, aureomycin or streptomycin (Lin *et al.*, 2015; Heys *et al.*, 2018), sometimes used in combination (Sharon *et al.*, 2011).

Tetracycline is a broad-spectrum antibiotic commonly used to treat a variety of bacterial infections (Zug & Hammerstein, 2015). In many studies, it is implicitly assumed that tetracycline treatment has no other effect than removing bacteria, but this is not always the case and effects attributed to the loss of gut microbiota might in fact be caused by

antibiotic toxicity toward the host. For example, Ballard and Melvin showed in 2007 that tetracycline treatment reduced mitochondrial efficiency and so decreased ATP production in *Drosophila simulans*. This could have a direct influence on traits such as fecundity or longevity, which may easily be confused with a "bacteria loss effect". Another study on pseudoscorpions (*Cordylochernes scorpioides*) found that sperm viability was reduced because of tetracycline, and that its effect could be passed down generations (Zeh *et al.*, 2012).

The cabbage root fly *Delia radicum* (Diptera: Anthomyiidae) is one of the most serious pest of *Brassica* plants within the temperate zone of the holarctic region (Finch, 1989). It has a relatively rich bacterial community for an arthropod (50-100 potential species), which has recently been described (Bili *et al.*, 2016). Amongst these bacterial taxa, this study highlighted the presence of one vertically transmitted intracellular symbiont: *Wolbachia sp*. In Brittany, the prevalence of *Wolbachia* in *D. radicum* varies a lot depending on the population studied. When the bacteria is present, the infection ranges from 5% to 10% in the wild, rises up to 55% in laboratory conditions, while some wild strains seem completely *Wolbachia*-free (unpublished work, but see Table 1 and Figure 2 in the General Discussion).

This work is the first aimed at assessing the effects of gut microbiota on the life history traits of *D. radicum*. To investigate the sole effects of gut symbionts, a *Wolbachia*-free strain was used to ensure that the possible effects observed would not be due to this endosymbiont but only to the modification of the gut community. Since an antibiotic treatment (tetracycline) was used to create symbiont-depleted individuals, a protocol spanning three generations was established, which allowed to discriminate the possible direct effect of tetracycline on the host (chemical effect) from its indirect effects (due to the partial or total loss of gut microbiota). Because tetracycline is a broad-spectrum antibiotic which was fed to individuals all their adult life, it is indeed most likely to have had a massive effect on the microbiote, although some taxa might have survived the treatment. However, our objective being to document the effect of a depleted microbiote and not to generate aposymbiotic (symbiont free) lines, no attempt was made to check for the total absence of symbionts post-treatment.

By comparing the offspring (and the grandchildren) of antibiotic-treated vs untreated *D. radicum* flies from the egg to the adult stage, we measured emergence rate, development time, sex ratio, adult size, survival without food and egg load in offspring females. Because the gut microbiota can modulate many aspects of host physiology, we expected the antibiotic treatment to largely affect life history traits and to have negative effects overall.

MATERIAL & METHODS

Insect culture

The *Delia radicum* strain studied here was originally reared from 300 adults emerged from pupae collected in 2015 in experimental broccoli fields near Le Rheu (Brittany, France, 48°07′16″N, 1°47′41″O). These flies were reared in the lab in a climate-controlled room (20 ± 1°C, 60 ± 10% RH, L16:D8) on swedes roots (*Brassica napus subsp. rapifera*) following a method derived from van Keymeulen *et al.* (1981). After emergence, individuals were placed in several rearing cages (Bug Dorm-4 Insect Rearing Cage, 47.5 x 47.5 x 47.5cm) with wet cotton as a water source and a 1:1:1 mix of sugar, milk powder and dietary yeast *ad libitum* as food.

No specific permissions were required for this experiment which did not involve endangered or protected species.

Antibiotic treatments

In this experiment, tetracycline hydrochloride powder (Sigma-Aldrich, CAS number: 64-17-5) was used as this product is freely soluble in water and thus, very convenient to use. Treated flies were fed with regular food (1:1:1 mix of sugar, milk powder and dietary yeast) but a water source containing dissolved tetracycline to a final concentration of 0.5 mg/mL (preliminary experiments showed that this concentration was the strongest one we could use without increasing the mortality of individuals treated) . Treated individuals were given tetracycline continuously during their whole adult lifespan.

Creation of the four lines with crossed treatments

The *D. radicum* lab stock was used to create four separate lines for our experiments to allow us to discriminate the direct effect of tetracycline (chemical toxicity) from its indirect effect (the possible loss of *Wolbachia*). Tetracycline was administered during two generations (G0 and G1) before measurements were made on the third one (G2) (Figure 1). All experiments were carried out in a climate-controlled room (20 ± 1°C, 60 ± 10% RH, L16:D8).

In our lab culture, 400 females and 400 males aged 1-3 days were sampled randomly in several insect rearing cages containing more than 3000 flies overall. Individuals from this generation zero (G0) were then randomly distributed in two different rearing cages (Bug Dorm-4 Insect Rearing Cage, 47.5 x 47.5 x 47.5cm) with a 1:1:1 mix of sugar, milk powder and dietary yeast *ad libitum* as food. The control cage was left untreated with mineral water as a water source, while the antibiotic cage contained mineral water with a concentration of 0.5 mg/mL tetracycline. Volvic® water was used in both cases because of its neutral pH and was renewed every other day to avoid oxidation of tetracycline. After 15 days, small pieces of swede roots were put in both cages for 48 hours for females to lay eggs. These eggs were collected and distributed on several swedes roots for them to develop according to the van Keymeulen *et al.* (1981) method. After 30 days, both groups of emerging adults (from control and treated parents) were again randomly separated in two cages, so that we had a total of 4 cages for generation 1 (G1) individuals. For each of the group, half of them were given regular water while the other one was treated with antibiotic. At this point, we thus had four different lines for G1: a control one where none of the generations were treated with the antibiotic (***C-C***); a line where only parents (G1) were treated (***C-A***); a line where only grandparents (G0) were treated (***A-C***); and a line where parents *and* grandparents were treated with tetracycline (***A-A***). After 15 days, small pieces of swede roots were put in the four cages for 48 hours for females to lay eggs. Then, 400 eggs were randomly sampled for each treatment amongst the several thousands that were collected. These 400 eggs were distributed by group of 10 eggs on 40 different turnip roots, large enough so that the larvae would have food *ad libitum* during their development. When generation 2 (G2) individuals emerged 30-45 days later, they were left untreated for all lines and life history traits measurements were made on them only (Figure 1).

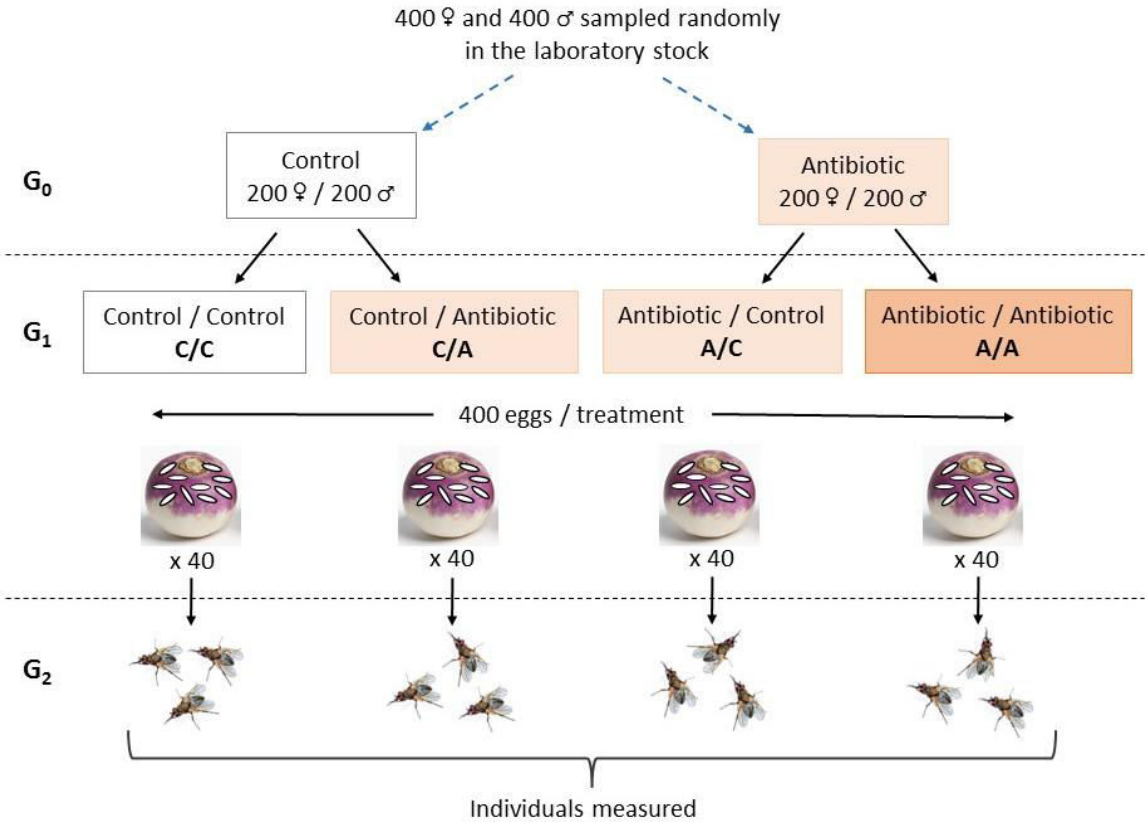


Figure 1. Schematic representation of the crossed treatments used to obtain four different lines of G2 individuals.

Life history traits

The relative fitness of G2 offspring was assessed by comparing several life-history traits:

- emergence rate (percentage of eggs yielding an adult);
- development time (duration from egg laying to imago emergence);
- sex-ratio (proportion of females);
- survival without food (100-104 emerging flies from each treatment were placed individually in cotton-plugged 9.5 mm x 2 mm Plexiglas tubes when they were less than 24h old. Each tube also contained a wet cotton ball as a water source, but no food. These individuals were then monitored daily at the same time and survival was therefore measured in days);
- adult size (157-191 randomly sampled dead flies were measured under a dissecting microscope fitted with a Euromex CMEX5 camera using the Image

Focus® software. As a proxy of body size, we measured the tibia on the right third leg of each fly with a ± 0.1mm accuracy);

- egg load (22-41 randomly sampled females were euthanized at 10 days of age, dissected under a binocular microscope, and mature eggs in the ovaries were counted.

Statistical analysis

The emergence rate (probability to develop from egg to adult) and sex ratio were analyzed using generalized linear models (GLMs) with respectively a quasibinomial and binomial error family and a logit link function. Turnip weight was used as a covariable:

*y ~ G0 treatment * G1 treatment + turnip weight*

Development time, adult size and survival without food were analyzed using linear mixed models (GLMMs) with a Gaussian error family and an identity link function.

For development time, sex was added as a fixed effect and turnip weight as a covariable. The pot where the fly developed was considered as a random factor as several offspring came out from the same pot:

*y ~ G0 treatment * G1 treatment + sex + turnip weight + (1|pot)*

To analyze adult size, development time was also used as a fixed factor:

*y ~ G0 treatment * G1 treatment + sex + development time + turnip weight + (1|pot)*

Survival without food was analyzed by including adult size in the model:

*y ~ G0 treatment * G1 treatment + sex + development time + size + turnip weight + (1|pot)*

Finally, egg load was analyzed using a generalized linear model (GLM) with a poisson error family and a log link function. Turnip weight and development time were used as covariables:

*y ~ G0 treatment * G1 treatment + development time + turnip weight*

After statistical modelling, pairwise comparisons between treatments were performed using least-squares means. The mean values obtained and their associated standard errors were used to make the graphs.

All statistics were performed with the R.3.0.2 software (R Core Team, 2017) using packages RVAideMemoire (Hervé, 2017), car (Fox & Weisberg, 2011) for GLMs, lme4 (Bates *et al.*, 2015) for GLMMs and emmeans (Lenth, 2018) for pairwise comparisons.

RESULTS

Emergence rate

The emergence rate of G2 is significantly influenced by the G1 treatment (Table 1). More specifically, the emergence rate is reduced by the tetracycline treatment of their parents (G1) when these parents had themselves untreated parents. Then, the probability to emerge is 37 % lower compared to control individuals (Figure 2). However, if grandparents (G0) have been treated, a further tetracycline treatment of their offspring (G1) does not reduce significantly the emergence rate of G2 individuals (Figure 2).

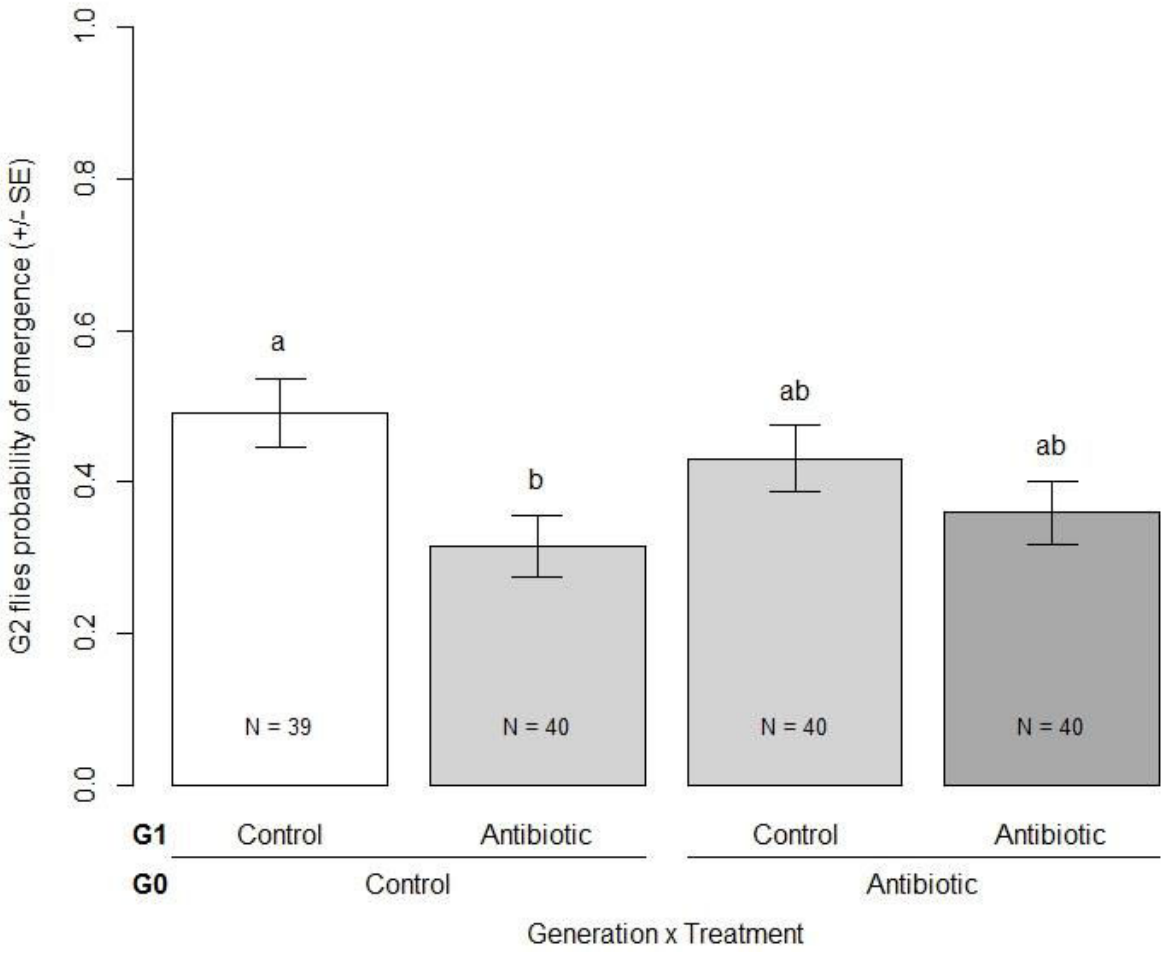


Figure 2. Crossed effect of antibiotic treatment and generation treated on the probability of emergence for *Delia radicum* generation 2 (G2) individuals. White: untreated for two generations, light grey: treated for one generation only (G0 or G1), dark grey: treated for both generations. Bars: standard error. N = number of replicates. Different letters indicate significant differences between treatments.

Table 1. Values and probabilities of GLM and GLMM tests conducted on fixed effects (G0, G1 and their interaction, sex and development time) in models computed for each trait. For all tests: df = 1. Significant effects are presented in bold.

Trait	Variable	*Statistic*	*P*
Emergence rate	G0	F = 0.012	0.911
	G1	F = 9.486	**< 0.01**
	G0 x G1	F = 1.698	0.194
Development time	G0	χ^2 = 6.942	**< 0.01**
	G1	χ^2 = 0.430	0.512
	G0 x G1	χ^2 = 1.651	0.199
	sex	χ^2 = 90.329	**< 0.001**
Sex ratio	G0	LR χ^2 = 0.011	0.917
	G1	LR χ^2 = 0.375	0.540
	G0 x G1	LR χ^2 = 0.001	0.989
Size	G0	χ^2 = 23.446	**< 0.001**
	G1	χ^2 = 7.842	**< 0.01**
	G0 x G1	χ^2 = 3.081	0.079
	sex	χ^2 = 11.647	**< 0.001**
	development time	χ^2 = 49.899	**< 0.001**
Survival	G0	χ^2 = 0.598	0.439
	G1	χ^2 = 14.411	**< 0.001**
	G0 x G1	χ^2 = 0.003	0.987
	sex	χ^2 = 20.372	**< 0.001**
	development time	χ^2 = 3.631	0.057
	size	χ^2 = 6.149	**< 0.05**
Egg load	G0	LR χ^2 = 0.02	0.895
	G1	LR χ^2 = 4.78	**< 0.05**
	G0 x G1	LR χ^2 = 49.90	**< 0.001**
	development time	LR χ^2 = 26.42	**< 0.001**
	size	LR χ^2 = 433.18	**< 0.001**

Development time

Surprisingly, the development time of G2 individuals is significantly affected only when the *grandparents* (but not the parents) have been treated with the antibiotic (Table 1, Figure 3). Then, G2 individuals take on average two days longer to develop than control ones. However, the development time of individuals whose *parents* were treated with tetracycline does not differ significantly from the control, whether their grandparents were treated or not. More precisely, these individuals have a "medium" development time, which does not differ significantly from any treatment.

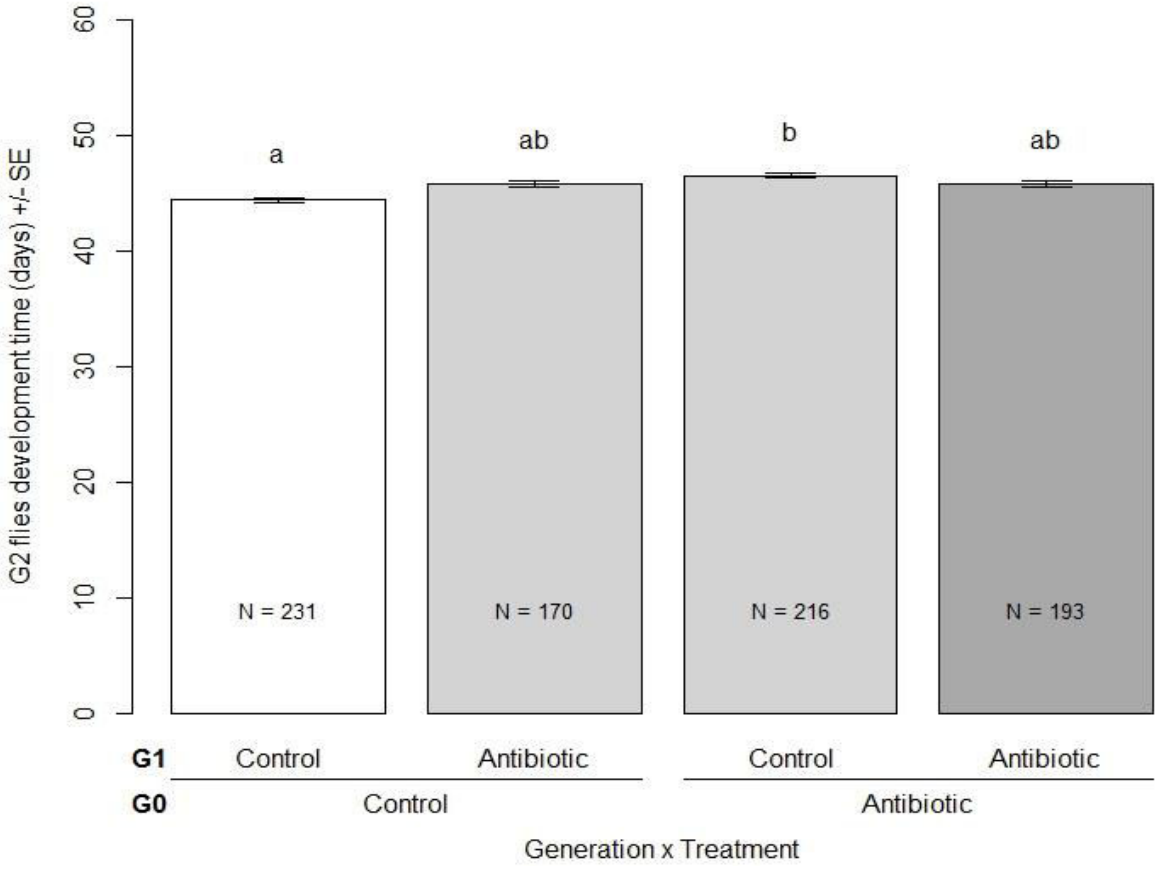


Figure 3. Crossed effect of antibiotic treatment and generation treated on the development time for *Delia radicum* generation 2 (G2) individuals. White: untreated for two generations, light grey: treated for one generation only (G0 or G1), dark grey: treated for both generations. Bars: standard error. N = number of replicates. Different letters indicate significant differences between treatments.

G2 development time is also strongly sex-dependent, males developing on average 2.5 days faster than females (χ^2: 90.329, df = 1, $P < 0.001$; males: 44.37 ± 0.34 days and females: 46.90 ± 0.35 days).

Size

The size of G2 individuals is influenced by the antibiotic treatment applied on G0 and G1, but not their interaction. It is also influenced by sex and development time (Table 1, Figure 4).

Surprisingly, as for development time, the size of G2 individuals is significantly modified only when their *grandparents* have been treated with tetracycline but their parents have *not* (Figure 4). Then, G2 individuals are slightly (4 %) but significantly larger than the fully untreated (C-C) control. On the other hand, the tetracycline treatment of G1 does not yield any effect, whether the G0 has been treated or not (Figure 4).

Besides, males have longer tibias (males: 2.003 ± 0.009 mm vs females: 1.975 ± 0.009 mm) and size is negatively correlated to development time: longer development times yield smaller individuals (Pearson's correlation: t = -9.0618, df = 596, p-value $P < 0.001$).

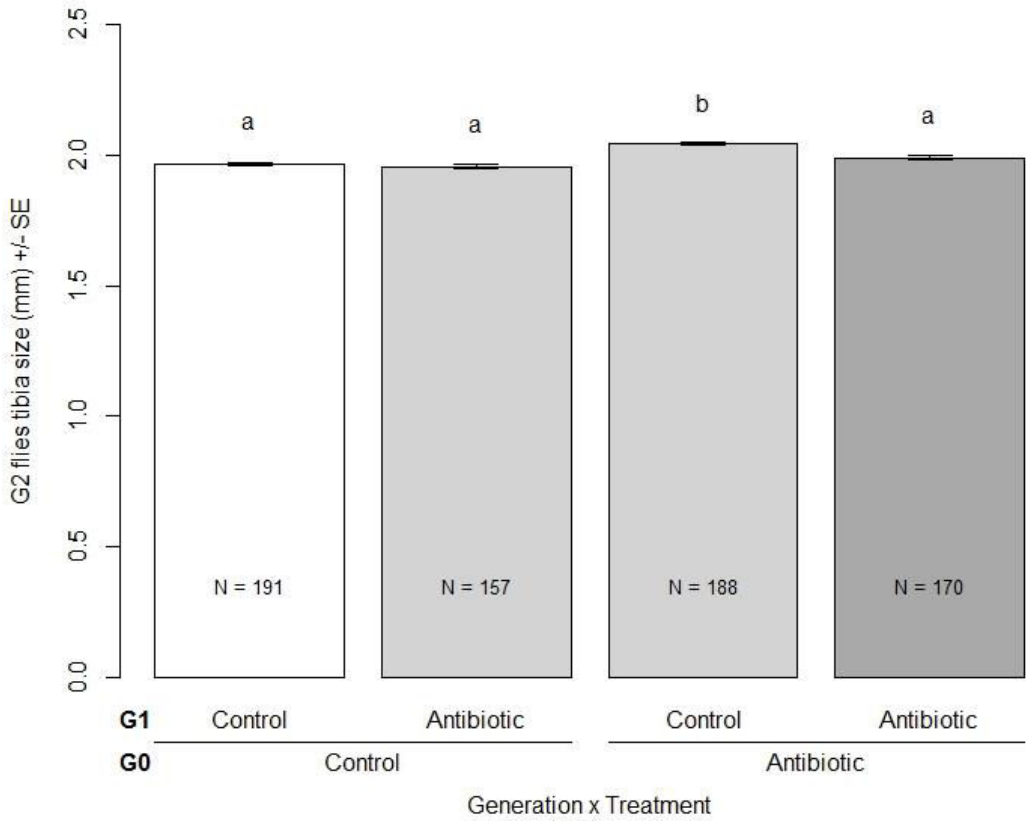


Figure 4. Crossed effect of antibiotic treatment and generation treated on tibia size for *Delia radicum* generation 2 (G2) individuals. White: untreated for two generations, light grey: treated for one generation only (G0 or G1), dark grey: treated for both generations. Bars: standard error. N = number of replicates. Different letters indicate significant differences between treatments.

Survival

The adult survival of G2 individuals in starving conditions (water but no food after emergence) is significantly reduced (by 8-10 %) when parents (G1) are treated with tetracycline, whether these parents had themselves treated parents or not (Table 1, Figure 5).

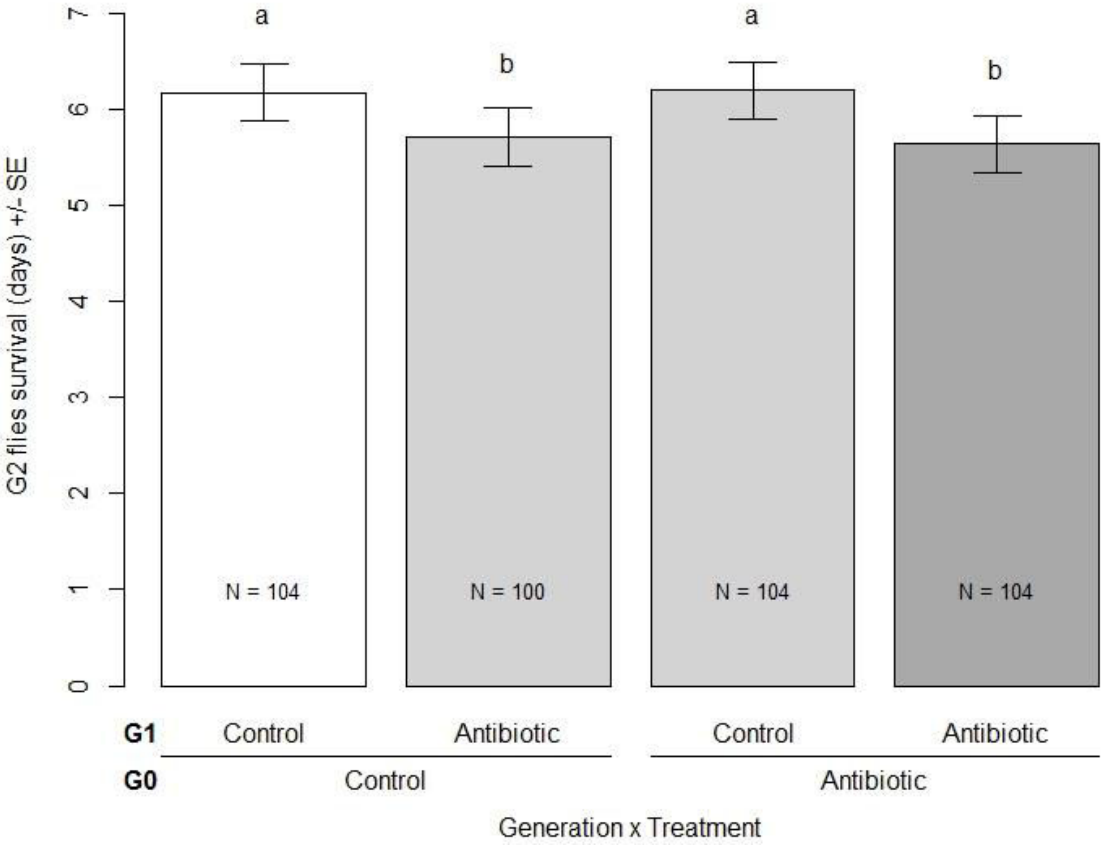


Figure 5. Crossed effect of antibiotic treatment and generation treated on survival to starving conditions for *Delia radicum* generation 2 (G2) individuals. White: untreated for two generations, light grey: treated for one generation only (G0 or G1), dark grey: treated for both generations. Bars: standard error. N = number of replicates. Different letters indicate significant differences between treatments.

Sex ratio

Sex ratio (expressed as the percentage of females) is not influenced by the treatment ($SR_{C\text{-}C}$ = 48.7 ± 4.1%, $SR_{C\text{-}A}$ = 46.4 ± 4.5%, $SR_{A\text{-}C}$ = 49 ± 4.1%, $SR_{A\text{-}A}$ = 46.9 ± 4.4%; see Table 1

for statistics) and is not significantly different from a balanced 50:50 sex ratio (Chisq < 0.47, df = 1, $P > 0.43$ for all treatments).

Egg load

The egg load of G2 females is significantly reduced by the tetracycline treatment of their parents (G1) but only when these parents had themselves untreated parents (Table 1). Then, G2 females have on average 28% less eggs in their ovaries than control individuals (Figure 6). However, if grandparents (G0) had been treated, a further tetracycline treatment of their offspring (G1) does not reduce significantly egg load in G2 individuals (Figure 6).

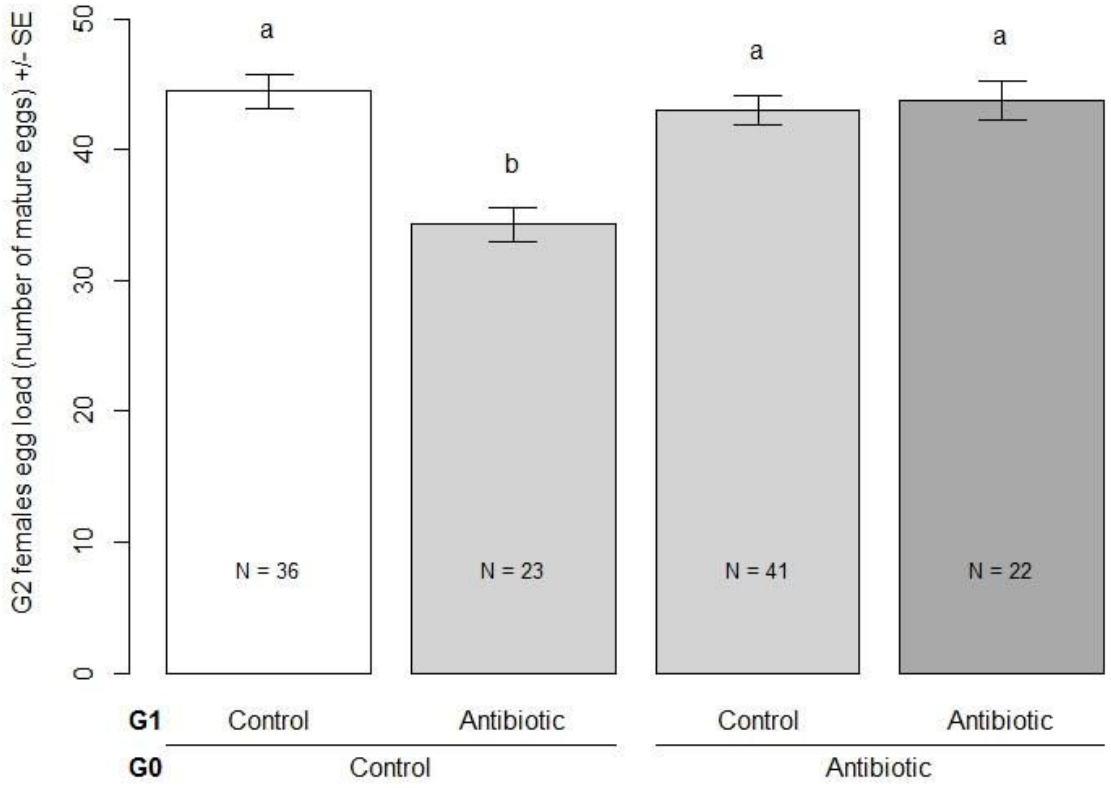


Figure 6. Crossed effect of antibiotic treatment and generation treated on egg load for *Delia radicum* generation 2 (G2) females. White: untreated for two generations, light grey: treated for one generation only (G0 or G1), dark grey: treated for both generations. Bars: standard error. N = number of replicates. Different letters indicate significant differences between treatments.

DISCUSSION

Overview

Our results demonstrate that depleting the gut microbiota of *Delia radicum* adults using a broad spectrum antibiotic treatment leads to multiple (and mostly negative) effects on life history traits in their descendance for at least two generations.

In this species, it is yet unknown if the gut microbiota is entirely acquired *de novo* from the environment or if it is partly inherited from the mother, in particular via bacteria coating the egg.

If the offspring acquired their microbiota from the environment only, this microbiota would build up while they are feeding and developing as a larva in the root regardless of their parent's gut status. This means that no matter the treatment, G2 individuals should all have the same gut microbiota since they were raised in identical conditions. Any difference observed between treatments would thus be due only to a maternal or grand-maternal effect (i.e. the phenotype of the parents/grandparents being affected by the antibiotic treatment). For example, a treated mother could produce smaller eggs, with less nutrients, which could lead to a reduced emergence rate. This deleterious effect of the antibiotic on G0/G1 could itself be due either to a toxic effect of the tetracycline (see "*Tetracycline: deleterious and beneficial effects*" below), or indirectly to the loss of symbiotic bacteria.

On the contrary, if the offspring obtained a significant part of their microbiota from their mother (most likely via the coating of the egg), differences observed between G2 individual groups would more probably be due to differences in the gut microbiota of G2 individuals themselves. This is because in case of an antibiotic treatment of the parental line, the inherited fraction of the microbiota would be devoid of all tetracycline-sensitive taxa but artificially enriched in tetracycline-insensitive ones.

In the following discussion, our results are interpreted according to these two scenarios. However, our data suggest overall that at least part of the microbiota of *D. radicum* is inherited maternally, and they outline the important role of such a microbiota in this species.

Because some of the traits we measured seem more influenced by a parental effect while others seem to depend more of the grandparent treatment, our discussion is structured accordingly.

Tetracycline: deleterious and beneficial effects

Tetracycline is a broad-spectrum agent, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites (Chopra & Roberts, 2001). This antibiotic works by entering the bacterial cell, binding to and blocking the 30S subunit of prokaryotic ribosomes, thus inhibiting translation and protein synthesis (Schnappinger & Hillen, 1996). Given the "bacterial" origin of mitochondria, tetracycline also targets mitochondrial translation and can impair mitochondrial function (Clark-Walker & Linnane, 1966; Moullan *et al.*, 2015). Indeed, several studies showed that treatment with doxycycline - a tetracycline derivative - disturbed mitochondrial proteostasis and metabolic activity, and induced widespread gene expression changes (Chatzispyrou *et al.*, 2015). In *Drosophila simulans*, tetracycline treatment has been shown to reduce mitochondrial efficiency and to lead to decreased ATP production, which could have a direct influence on fecundity or longevity (Ballard & Melvin, 2007). On the other hand, doxycycline – an antibiotic belonging to the family of tetracyclines – also proved beneficial effects on some physiological aspects such as an increased motility in nematodes and flies, and an extended lifespan of worms nematodes. However, at the same time, treated models showed developmental delay and physiological impairment related to body size and fecundity (Chatzispyrou *et al.*, 2015).

Together, these findings underline the large effects tetracycline can have on mitochondrial function and whole-body physiology, as well as the indirect effects that loss of bacterial symbionts can cause, and could partly explain the negative impact of this treatment we found in our study.

Efficiency of tetracycline treatments

Tetracycline has been a widely used antibiotic because of its relatively low toxicity and broad spectrum of activity. Tetracycline-resistant bacterial were first isolated in 1953

from populations of *Shigella dysenteriae*, a bacterium which causes bacterial dysentery. Since then, tetracycline-resistant bacteria have been found in an increasing number of species and genera; in pathogens, opportunistic and normal flora species (Roberts, 1996). In a recent study, Lin *et al.* (2015) evaluated the effects of five antibiotics (rifampicin, ampicillin, tetracycline, streptomycin sulfate and chloramphenicol) on the gut bacterial diversity of the diamondback moth (*Plutella xylostella*). They found that even though species diversity was significantly reduced because of the antibiotic treatments, gut bacteria could not be completely removed when treating moths with oral antibiotics. Moreover, in regard to antibiotics concentrations, the effectiveness of gut bacterial elimination was not obvious when the concentration was below 1 mg/mL in their experiment. Since we used a 0.5 mg/mL here, we assume that probably not all of the microbiota community was eliminated during our treatments. Even though flies were given tetracycline during their whole lifespan, they were therefore probably not aposymbiotic. We expect however that their gut microbiota consisted mostly of tetracycline-insensitive taxa having survived the treatment and multiplied to colonize the gut. This probably led to a very different microbiota in treated individuals (compared to control ones), which was potentially transmitted to the next generation.

Traits with a parental effect

Egg load, survival in starving conditions and emergence rate were only influenced by the treatment of parents (G1), and we can distinguish two cases: (i) the parents were the first to be treated (***C-A***) or (ii) grandparents had already been treated before (***A-A***).

When the phenotype observed in G2 individuals from the ***C-A*** line is inferior to the control line (***C-C***), we can suspect either a direct toxic effect of tetracycline on the parents or an indirect negative effect on the parents due to the loss of beneficial symbiotic bacteria in their gut (with the added possibility of a modified G2 gut microbiota if part of it is inherited). The toxic effect of tetracycline can however be dismissed if the ***A-A*** treatment does not differ from the ***C-C*** one. Of course, negative effects of toxicity and microbiota perturbation could add up, which would worsen the genotype of ***A-A*** G2 flies.

The only case where the egg load of G2 females is reduced is when their parents are treated with tetracycline, but only when these parents had themselves untreated parents.

This effect on the ***C-A*** line confirms that treating wild-type parents with a large spectrum antibiotic impairs their inclusive fitness, here by reducing that of their offspring. Moreover, the fact that this ***C-A*** line is the only one with a decreased phenotype allows us to conclude that it is the perturbation of the gut microbiota of the mother (and not a direct toxicity of tetracycline towards her) which influences the fitness of its offspring. Moreover, the fact that tetracycline does not have a significant effect on ***A-A*** offspring indicates that either the microbiota of their parents was already absent when the antibiotic was applied or that it consisted mostly of tetracycline-insensitive taxa having survived the treatment of the grandparents. Together, these results thus strongly suggest that a functionally important part of the microbiota of *D. radicum* is inherited.

The survival of both C***-A*** and ***A-A*** offspring to starving conditions is significantly but similarly impacted (survival is reduced by 8-10 %) compared to control ***C-C*** offspring. It is therefore not possible here to exclude a direct toxic effect of tetracycline on the mother. However, it is difficult to imagine that during their development in the unsterilized roots, larvae would not acquire any bacteria from their environment, so the effect found could still be explained by the adult mother losing the tetracycline-sensitive part of the microbiote it had acquired as a larva.

Finally, we observed that the emergence rate of offspring was reduced when parents were treated with tetracycline, but only if the grandparents were not treated before. By the same reasoning used to discuss fecundity (egg load) results, we are then led to conclude that at least a significant part of the microbiota necessary for a normal emergence rate is maternally inherited.

Traits with only a grandparental effect

Size and development time were significantly influenced by the antibiotic treatment of grandparents (G0), but surprisingly not by the treatment of parents (G1).

G2 individuals were significantly larger when their *grandparents* had been treated with tetracycline but their parents had *not.* This results allows us to conclude again that the microbiota of *D. radicum* is at least in part inherited. Indeed, we would have the same values for ***C-C*** and ***A-C*** otherwise. Here we can dismiss any growth-enhancing effect of the antibiotic (otherwise ***A-A*** offspring would be significantly larger than ***C-C*** ones).

Individuals are therefore larger when their grandparents have been exposed to antibiotic because of a change in the G0 microbiota which has a positive influence on growth and has been inherited by G1 individuals. The fact that the ***A-C*** treatment outperforms all others for that trait suggests that the treatment of the grandparents followed by an untreated generation has yielded (temporarily?) a unique microbiota pattern which has a favorable impact on size. These bacteria might help the flies to better assimilate some essential nutrients or vitamins, as shown before in other insect species (reviewed in Flint *et al.*, 2012 and Engel & Moran, 2013).

Like size, development time is significantly modified when the *grandparents* receive antibiotic, but only if the parents are not treated afterwards. In this case, development time is approximately 2.5 days longer, which is not desirable especially for male flies. Indeed, in this species, males emerge on average 2.5 days before females. This very common phenomenon in insects is called protandry and allows precocious males to wait and be first to mate with emerging females from the same patch (Godfray, 1994), which increases their chance of paternity (Simmons et *al.*, 1994). Here, this lengthening of development time will be detrimental compared to males with untreated microbiota.
However, unlike in the case of size, the development time of ***A-C*** offspring is not significantly different from ***A-A*** ones, which does not allow to rule out a direct toxicity of tetracycline on the mother.

CONCLUSION

In this study, we found that an antibiotic treatment of *D. radicum* adults leads to multiple and mostly negative effects on life history traits in their offspring, some of which can happen two generations after treatment. While some of our results do not allow to exclude a direct toxicity of tetracycline on the parents, our data suggest that most of the life history traits measured are modified because of perturbations of the gut microbiota of the adults treated, and that this microbiota is at least partially inherited maternally. Amongst our four treatments, the offspring of the ***C-A*** line are the most severely impacted, with three out of five life history traits being degraded (emergence rate, survival without food and egg load). The fact that the ***A-A*** line is not significantly affected in some cases allows to conclude that the indirect effect of gut microbiota loss has a larger role than any

putative toxic effect of tetracycline itself. These results thus confirm a beneficial role of the "wild-type" gut microbiome in this species as it has already been shown. Indeed, a recent study showed that the cabbage root fly larval gut microbiome was capable of degrading isothiocyanates, toxic compounds emitted by brassicaceous plants as a defense mechanism (Welte *et al.*, 2016). Moreover, since some insect gut microbes participate in digestion, the drastic gut flora quantitative and qualitative change after antibiotics feeding is indeed likely to damage digestive functions, causing a reduced performance of individuals treated which is large enough to be detected in their offspring and, in some case, their grandchildren. In this study, we do not assume necessarily a total destruction of the microbiote post treatment but suggest instead that some tetracycline-insensitive bacteria taxa might actually have not only survived but multiplied to occupy the niches liberated by the antibiotic treatment. By mass sequencing of 16S DNA amplicons, we are now planning to describe and compare the richness and diversity of gut microbiota in our four kinds of treatments. With predominant species of bacterial strains identified for each one of them, it would then be possible to identify the contribution of a specific bacteria to a specific phenotype. Also, sequencing would allow us to confirm that tetracycline is not sufficient on its own to create an aposymbiotic line. In this case, another perspective allowing an easier interpretation would be to manage a complete symbiont elimination in further studies, by using a combination of antibiotics. However, the question of whether *D. radicum* would survive the total loss of its gut symbionts remains open.

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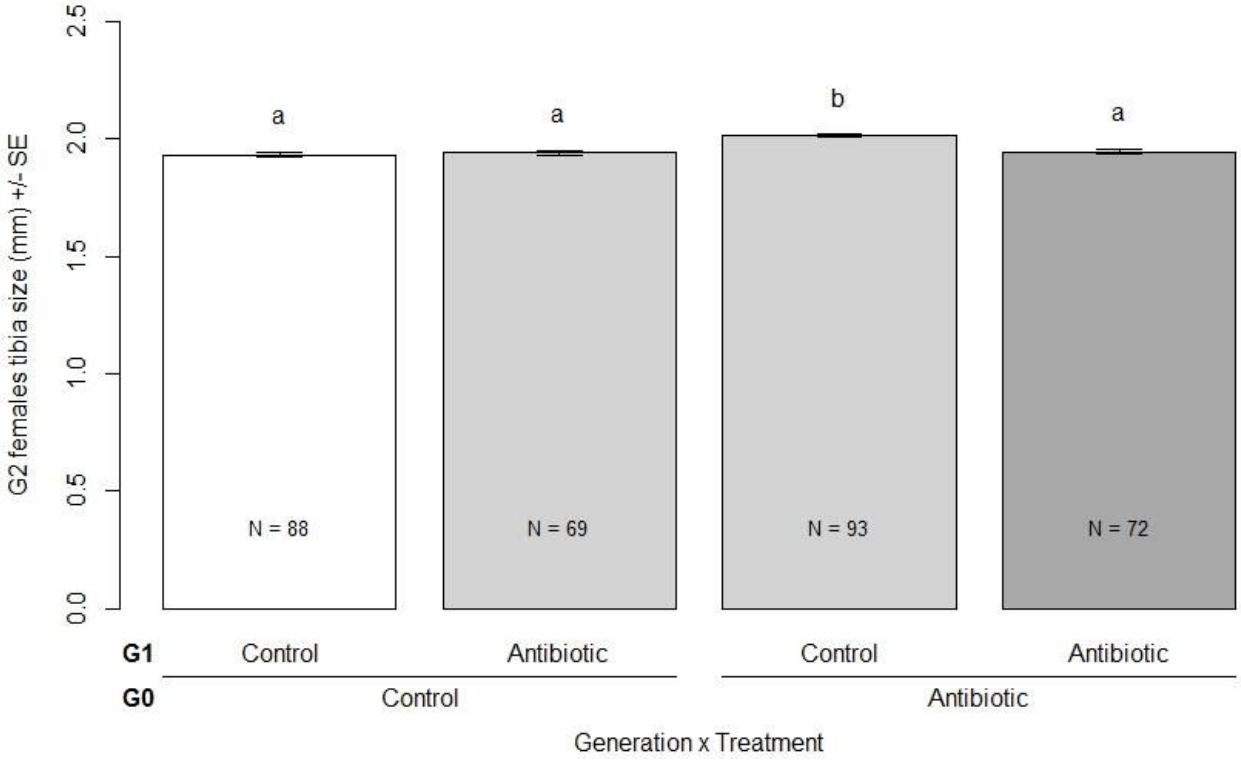
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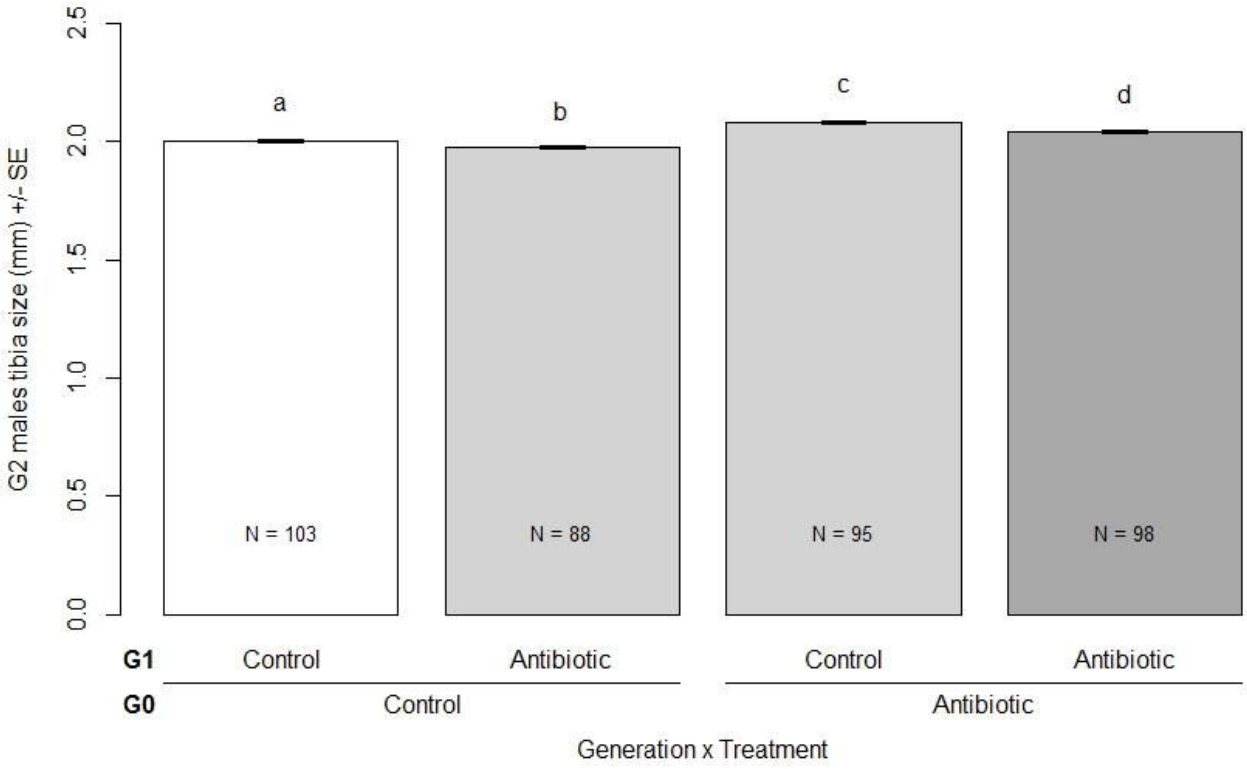
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APPENDIXES

In our study, size was not significantly influenced by the sex of individuals in our model, which is why size was presented and interpreted for both sexes at a time. However, since the size can influence survival, but also fecundity in females, the data for adult size is presented here for males and females, separately, for information purpose.



Appendix 1. Crossed effect of antibiotic treatment and generation treated on tibia size for *Delia radicum* generation 2 (G2) females. White: untreated for two generations, light grey: treated for one generation only (G0 or G1), dark grey: treated for both generations. Bars: standard error. N = number of replicates. Different letters indicate significant differences between treatments.



Appendix 2. Crossed effect of antibiotic treatment and generation treated on tibia size for *Delia radicum* generation 2 (G2) males. White: untreated for two generations, light grey: treated for one generation only (G0 or G1), dark grey: treated for both generations. Bars: standard error. N = number of replicates. Different letters indicate significant differences between treatments.

CHAPTER 3

Influence of the symbiont
Wolbachia on
plant-insect interactions

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Influence of the symbiont *Wolbachia* on plant-insect interactions

Context

Against herbivorous insects, plants have developed various chemical defense mechanisms that can be constitutive (i.e. present at all times) or induced (i.e. activated after an attack). These defenses can be direct and target the herbivore itself or indirect by attracting some of its parasitoids/predators. These mechanisms must constantly change in evolutionary time as insects constantly evolve new strategies to bypass them.

Recently, it has been discovered that *Wolbachia*, an endocellular insect symbiont, could play a role in the plant-insect interaction. Indeed, this symbiont seems able to interfere directly with plant defenses by manipulating plant hormones, and thus regulating some signaling pathways. From an evolutionary point of view this makes sense, since *Wolbachia* is only maternally transmitted via the cytoplasm of the egg and shares the same interest as its host in a reduced plant defense.

The cabbage root fly *Delia radicum* is an oligophagous insect specialist of the Brassicaceae family. Root-feeding by *D. radicum* is very well-known to modify the levels of plant chemical defenses, whether they are direct (glucosinolates) or indirect (volatiles). The fly microbiome has been described recently and revealed the presence of the symbiont *Wolbachia sp.,* an intracellular bacterium which possible influence on brassicaceous plants defense against the cabbage root fly has never been studied.

Approach

In this study, we investigated whether *Wolbachia* could modify chemical responses to phytophagy in oilseed rape (*Brassica napus*) by comparing the plant defenses following an attack by larvae of *D. radicum* infected or not by this intracellular symbiont. To do so, we induced *B. napus* roots by infesting them either with *Wolbachia*-infected *D. radicum*

larvae (*Wolbachia*-plus or WP) or by symbiont-free larvae (*Wolbachia*-minus or WM). Apart from their infection status, the two available *D. radicum* laboratory stocks had the same genetic background. Following induction, we measured and compared glucosinolate profiles and volatile blends between both treatments.

A first hypothesis was that plant responses could be affected directly by *Wolbachia* modifying the saliva chemistry of *D. radicum*, because this bacterium has been observed in the salivary gland cells of other fly species. A second hypothesis was that the endosymbiont could be able to modify plant signalling-pathways indirectly via the modification of its host's gut microbiota, which might modify in particular the chemistry of its host' feces. In any case, we expected different plant responses depending on the presence/absence of *Wolbachia*. We used plants bearing no attack as further controls (CON treatment).

Results

Ten different glucosinolates were detectable in our samples overall; five were present in roots and leaves, four only in the roots and one only in the leaves. Glucosinolates levels were significantly influenced by *Wolbachia* infection but in a different manner for each organ; with almost all groups differing significantly from each other (except WP roots *vs* WM roots and CON leaves *vs* WM leaves).

Attacked plants had lower root glucosinolate concentrations, irrespective of *Wolbachia* infection – except for one indole glucosinolate which was significantly less concentrated in control roots. Within our sample of attacked plants, concentrations were always the lowest in plants attacked by *Wolbachia*-infected larvae.

Plants attacked by *Wolbachia*-free larvae had the same leaf glucosinolate content as controls. However, leaf glucosinolate content decreased significantly in plants attacked by *Wolbachia*-infected larvae, particularly for the aliphatic compounds progoitrin, gluconapin and glucobrassicanapin.

Fifteen and thirteen different volatiles were detectable in our samples on day 1 and day 3, respectively, but treatments did not differ significantly.

The key result in this study is the finding that the endocellular symbiont of an herbivore can indeed influence the defense reaction of its host plant. Here, when plants were attacked with *Wolbachia*-infected larvae, we observed a decrease in aliphatic glucosinolates in the leaves, and consequently, a probable decrease of associated volatiles isothiocyanates. Such a change could be advantageous for *D. radicum* because these compounds are olfactive cues that can be used by *D. radicum* natural enemies to locate their host or prey. Likewise, a decrease in glucosinolates in the leaves could profit *D. radicum* because leaf glucosinolates stimulate oviposition in this species; this decrease might thus discourage competitors to lay on the same plant. Our study confirms that the rich field of insect-plant interactions studies becomes even richer when taking intracellular microorganisms into account.

Limits

Our number of replicates per treatment was limited (10 plants per treatment for glucosinolates and 5-9 plants per treatment for volatiles), which may not have supplied sufficient power to detect some differences, especially for the volatiles experiment, where some interesting trends could be observed but were not significant. This small number of replicates is due to the original experiment being based on more treatments, with 70 plants in total. Unfortunately, we decided to exclude some of these treatments from our analysis as they were not as flawless as we wished.

Another limit was that, although we measured glucosinolate concentrations in roots and leaves, the volatiles were only collected for the roots. Some of our conclusions could have been enriched by having the leaf volatiles data as well because leaf volatiles are particularly efficient to recruit flying parasitoids.

Perspectives

All dry matter samples we had left have been kept in a freezer in the laboratory. A further study focusing on gene expression in each treatment would thus be possible and very interesting to complete our results.

This work would also be nice to replicate with more repetitions per treatment, as we suspect that some effects might have "slipped under the radar" by lack of statistical power. Also, to account for the effect of quantitative feeding by larvae, it would be interesting to include the number of larvae deposited on the plant as a variable.

In this chapter you will find...

Besides the study described above, you will find at the beginning of this chapter a brief review of some of the plant chemical defenses against herbivores, and their modes of action.

Mini-review:
Plant chemical defenses against herbivores

Herbivory is the most common feeding strategy in insects even though plant tissues are challenging to consume and digest as they can contain a diversity of enzyme inhibitors or toxic chemicals (Schoonhoven *et al.*, 2005). Indeed, even though plants cannot "run away" from their predators, they are not passive victims of phytophagous insects. During their evolution, they have developed two different resistance modes: constitutive resistance, present at all times and expressed independently from stresses; and induced resistance, activated only when the plant is attacked or injured (Zhang *et al.*, 2008). In response, many insects have developed resistance or tolerance to plant defenses and are thus able to feed on them anyway. Therefore, an evolutionary "arms race" is taking place, where plants evolve new defense mechanisms - especially by the synthesis of new compounds that are toxic or anti-nutritious for insects - while insects evolve new strategies to bypass them. Ehrlich & Raven (1964) suggested a model of coevolution that directly connected plant and insect diversifications after highlighting symmetrical phylogenies between butterflies and plants. However, a recent paper showed an apparent asymmetry in the interactions between plants and herbivores, and proposed that instead, herbivores may be evolutionarily "chasing" plants, feeding on species for which they have preadaptations (Endara *et al.*, 2017). In any case, plant defense chemicals may become a message which interpretation by the insect will depend on its level of specialization: specific defense chemicals will act as a deterrent for generalist insects, unable to handle them, but will on the contrary be a reliable host plant signal for specialist phytophagous species (Ali & Agrawal, 2012).

When an insect attacks a plant, it elicits a biochemical cascade of events leading to various changes in plant physiology. Plants can perceive insect attacks (i) mechanically (tissue damage caused by mandibles or stylets), in which case elicitors will be produced by the plant wound itself; but also (ii) chemically via plant receptors detecting molecules present on the insect cuticle or in their oral secretions, saliva or feces. All these cues result in the activation of plant defense pathways (Felton & Tumlinson, 2008).

Phytohormones are endogenous signal molecules active at very low doses and controlling plant physiology and development. They also mediate plant responses to biotic and

abiotic stress and induce or suppress the expression of genes involved in the synthesis of enzymes, pigments and metabolites (Tsavkelova *et al.*, 2006; Wasternack & Hause, 2013). Many plant hormones have been described but three of them have been extensively studied and shown to play major roles in regulating plant defenses and plant-insect interactions: Jasmonic Acid (JA), Salicylic Acid (SA) and Ethylene (ET) (Kessler & Baldwin, 2002; Pieterse & Dicke, 2007; Erb *et al.*, 2008, 2012; Dicke & Baldwin, 2010; Pieterse *et al.,* 2012). JA and ET mostly act synergistically, are generally associated with plant defense against chewing herbivores and necrotrophic[2] pathogens, and activate the expression of both direct and indirect defenses. SA is usually linked with activating defense responses against biotrophic[1] pathogens and sap-feeding insects (Kessler & Baldwin, 2002; Grant & Lamb, 2006; Koornneef & Pieterse, 2008; Rani & Jyothsna, 2010; Shivaji *et al.*, 2010; War *et al.*, 2011). In nature, however, plants often deal with simultaneous or subsequent invasion by multiple aggressors, which can influence the response of the host plant (van der Putten *et al.*, 2001; Bezemer & Van Dam, 2005). JA and SA usually act antagonistically which means that JA can inhibit SA-signalling pathways, and vice-versa (Niki *et al.*, 1998). However, much evidence has shown that JA and SA can also sometimes have synergistic interactions (Salzman *et al.*, 2005; Beckers & Spoel, 2006). Once these phytohormones activate their signalling pathways, several defense mechanisms come into play.

First, herbivory can increase the levels of specialized (or secondary) metabolites used in direct defense (Hopkins *et al.*, 1999; van Dam & Raaijmakers, 2006; Pierre *et al.*, 2012). Specialized metabolites are compounds that are not essential to the living cells. In contrast to primary metabolites, they are not found in every species, but are often associated with distinct taxonomic groups. As a result, they account for most of the molecular diversity of living organisms (Tissier *et al.*, 2014). For example, glucosinolates (i.e., mustard oils) are specialized metabolites produced by sixteen families of dicotyledonous angiosperms including a large number of edible species (Fahey *et al.*, 2001). Glucosinolates (GSLs) are a widely studied class of plant chemical compounds with

[2] Necrotrophic pathogens derive energy from killed cells; they invade and kill plant tissue rapidly and then live saprotrophically on the dead remains. Biotrophic pathogens derive energy from living cells, are found on or in living plants, can have very complex nutrient requirements and do not kill host plants rapidly. Some pathogens have an initial period of biotrophy followed by necrotrophy, and are called hemibiotrophs.

a large structural diversity. They are found in the seeds, roots, stems and leaves. Over 130 GSLs have been identified to date, mainly in species belonging to the Brassicaceae family (Fahey *et al.*, 2001; Agerbirk & Olsen, 2012). Broadly, GSLs can be divided into three classes based on the structure of different amino acid precursors: (i) aliphatic GSLs, derived from methionine, isoleucine, leucine or valine, (ii) aromatic GSLs, derived from phenylalanine or tyrosine, and (iii) indole GSLs, derived from tryptophan (Halkier & Gershenzon, 2006; Radojčić Redovniković *et al.*, 2008). These three major types of GSLs often respond differently to herbivory (Textor & Gershenzon, 2009).

Intact GSLs are stored in the vacuoles of plant cells and can be found in any aboveground or belowground organ (van Dam *et al.*, 2009). Upon tissue damage and cell rupture (e.g. herbivory), GSLs are released and are mixed with the enzyme myrosinase – also stored in specific cells and apart from GSLs – that will hydrolyze them (Vig et al., 2009), setting off a "mustard oil bomb" (Ratzka *et al.*, 2002). More precisely, myrosinase converts these GSLs into various toxic degradation products such as isothiocyanates (ITCs), which are volatile. These degradation products are toxic for generalists and specialists; however, some specialists can prevent their formation while generalists usually cannot (Agrawal & Kurashige, 2003; Hopkins *et al.,* 2009). ITCs will act as a deterrent for generalists (unable to handle them) but will on the contrary be attractive and stimulating compounds for specialist herbivores (Ali & Agrawal, 2012). However, these volatile ITCs emissions can also attract and stimulate specialist natural enemies of phytophagous insects (Lamb, 1989; Bartlet, 1996; Murchie *et al.*, 1997). At first, plant-induced responses were mostly investigated in aboveground communities, but a broader picture has now emerged, with studies connecting above- and belowground compartments (van der Putten *et al.*, 2001; van Dam *et al.*, 2003). Notably, root-feeding was shown to induce both a local (root) and a systemic (foliar) increase in levels of GSLs in wild *Brassica* species (Bezemer & van Dam, 2005; van Dam & Raaijmakers, 2006). This means that defense compounds found in roots are also present in leaves, suggesting that root and leaf induction involves similar pathways.

As a second line of defense, which is activated only in response to herbivory, plants are known to emit a complex blend of volatile organic compounds called Herbivore Induced Plant Volatiles (HIPVs). They differ from volatile organic compounds (VOCs) naturally released by intact plants or elicited by mechanical damage (Turlings *et al.*, 1995;

Takabayashi & Dicke, 1996). HIPVs function mostly as an indirect defense for the plant because they do not target the herbivore directly but attract its natural enemies such as predators or parasitoids (Price *et al.*, 1980; Turlings *et al.*, 1995; Vet & Dicke, 1992; Dicke, 1999; Vet, 1999). These induced blends of plant volatiles can be emitted locally at the site of damage, or systemically by undamaged tissues of affected plants (Heil & Ton, 2008). More than 200 HIPVs have been identified already, mainly terpenoids, fatty acid derivatives, phenylpropanoids and benzenoids. Some are biosynthesized only when the plants are attacked, while others are also naturally emitted by undamaged plants, but in much lower quantities than by damaged ones (Dicke & Baldwin, 2010). Remarkably, the response of a given plant species may vary according to the herbivore taxonomic identity. Indeed, some plants can recognize which species is feeding on them and adapt their response accordingly. This leads to the production of specific qualitative and quantitative mixtures of volatile compounds (Dicke *et al.*, 2009), which results in highly reliable taxonomic cues allowing natural enemies to locate their precise host or prey (Neveu *et al.*, 2002; Dicke *et al.*, 2003; Soler *et al.*, 2007; Pierre *et al.*, 2011). For instance, the specialist parasitic wasp *Cardiochiles nigriceps* prefers plant odors emitted by tobacco plants infected with its host, *Heliothis virescens*, vs non infected ones. Better, when the parasitoid has the choice between plants infested with its host and plants infested by a non-host but related species (*Heliothis zea*), the wasp can still locate the plants attacked by its favorite prey thanks to the specific volatile cues (De Moraes *et al.*, 1998).

To counteract plant defense mechanisms, herbivorous insects have developed several strategies. Particularly, they can manipulate plant hormone biosynthesis to lower plant defenses (Giron *et al.*, 2013). More surprisingly, there is growing evidence that insect microorganisms are important 'hidden players' in these insect-plant interactions, and participate in plant manipulation to the benefit of their insect host. Such insect mutualist symbionts can interfere directly with plant defenses by regulating phytohormone-signalling pathways (Kaiser *et al.*, 2010; Frago *et al.*, 2012; Body *et al.*, 2013; Giron *et al.*, 2013, 2016, 2017; Sugio *et al.*, 2015). For example, when feeding, the Colorado potato beetle *Leptinotarsa decemlineata* releases bacteria in its oral secretions that disrupt phytohormone expression and suppress induced plant defenses (Chung *et al.*, 2013). Intracellular insect symbionts have also been shown to play a part in insect-plant interactions. In the whitefly *Bemisia tabaci*, the saliva of individuals harboring the

symbiont *Hamiltonella defensa* is able to suppress JA-related defenses although *H. defensa* is restricted to specialized bacteriocyte cells apparently unrelated to salivary glands (Su *et al.*, 2015). However, the presence of insect symbionts can also be exploited by the plant to better detect the insect host. For example, in the aphid-*Buchnera* system, a symbiont protein delivered in the insect saliva is recognized by the plant and activates its defenses (Barr *et al.*, 2010). However, in this system, *Buchnera* has been an obligatory symbiont of aphids for millions of years, which probably explains why the plant adapted to recognize it.

Endocellular symbionts – whether they are obligate or facultative – could theoretically participate in the plant-insect dialogue in two different ways. They could directly produce elicitors reaching the plant via the salivary glands or the feces of their insect host (indeed, some bacteria are able to synthesize phytohormones on their own - Costacurta & Vanderleyden, 1995; Stes *et al.*, 2011). They could also modify gut microbiota communities (Simhadri *et al.*, 2017), which might also change the chemistry of the saliva or feces of their host. If endosymbionts can influence the plant-host relationships, it is therefore justified to test the effect of their presence or absence, especially in insect/plant pairs for which the direct and indirect plant defenses are well-known.

Accordingly, we investigated in the following study the possible impact of an endocellular symbiont, *Wolbachia*, on a plant-insect interaction that has been extensively studied: the cabbage root fly *Delia radicum* feeding on a Brassica species, *Brassica napus*.

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In preparation for Journal of Chemical Ecology / Ecology

Insiders reach out: an insect intracellular symbiont modifies the defense of a plant to phytophagy

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Abstract: In the *Brassica* plant species, specialized metabolites such as glucosinolates (GSLs) constitute a direct defense against phytophagy because, upon tissue damage, they will be converted in toxic compounds called isothiocyanates (ITCs). Intact plants also naturally emit volatile organic compounds (VOCs), but herbivory can modify these volatiles blends, releasing a new complex bouquet called Herbivore Induced Plant Volatiles (HIPVs). They function as an indirect defense for the plant since they do not target the herbivore directly but will attract its natural enemies or competitors. The interactions between the cabbage root fly (*Delia radicum*) and *Brassica* plants have been extensively studied and this insect is very well-known to trigger these two types of plant defenses. The *D. radicum* studied here carries the symbiont *Wolbachia*, which has been shown to manipulate plant defenses for the benefit of its insect host in other species. In this study, we investigated whether *Wolbachia* could modify the insect-plant dialogue between *D. radicum* and oilseed rape (*Brassica napus*). To do so, we compared the GSLs concentrations and volatile emissions following phytophagy by *D. radicum* larvae infected or not by this symbiont. When plants were attacked by infected larvae, aliphatic GSLs concentrations decreased in the leaves. Since leaf GSLs are used as oviposition stimulants in *D. radicum*, *Wolbachia* could thus increase the fitness of its host by decreasing the cues that could be used by other conspecifics, and therefore, avoid

competition. Likewise, less GSLs in the leaves means less emitted ITCs in the case of simultaneous aboveground herbivory, which would favor *D. radicum* by reducing the attraction of its natural enemies. HIPVs blends did not differ significantly between treatments. This study shows the potential of *Wolbachia* to influence plant-insect relationships, which confirms that this rich field of study should now take endocellular symbionts into account.

Keywords: bacterial symbiont, *Wolbachia*, *Delia radicum*, cabbage root fly, *Brassica*, glucosinolates, volatiles, HIPV, isothiocyanates

INTRODUCTION

During their evolution, plants have developed a vast array of chemical defenses against phytophagous insects (Zhang *et al.*, 2008). Plants can confront insects both directly by affecting their plant choice, survival and reproduction, and indirectly by attracting their natural enemies such as predators or parasitoids. These defenses can be constitutive (i.e. present constantly and expressed independently from biotic stresses) or induced (i.e. activated only after an attack) (War *et al.*, 2012). The key role of specialized metabolites in all these defenses has been documented in many plant insect interactions (Bennett & Wallsgrove, 1994; Rosenthal & Berenbaum, 2012; Zaynab *et al.*, 2018).

Biochemical defenses are not static mechanisms but constantly change in evolutionary times as plants and insects are involved in an evolutionary "arms race" where plants evolve new direct and indirect defense mechanisms while insects evolve new strategies to bypass them. While this biochemical "arms race" has been proposed for a long time (for a seminal paper, see Erlich & Raven, 1964), there is now growing evidence that insect microorganisms are important "hidden players" in these insect-plant interactions, and participate in plant manipulation, generally to the benefit of their insect host. Such insect mutualist symbionts can interfere directly with plant defenses by regulating phytohormone-signaling pathways involved in induced defenses (Kaiser *et al.*, 2010; Frago *et al.*, 2012; Body *et al.*, 2013; Giron *et al.*, 2013, 2016, 2017; Sugio *et al.*, 2015). More surprisingly, some *intracellular* insect symbionts have also recently been shown to play a part in insect-plant interactions. In the whitefly *Bemisia tabaci*, the saliva of individuals harboring the symbiont *Hamiltonella defensa* is able to suppress JA-related defenses although *H. defensa* is restricted to specialized bacteriocyte cells apparently unrelated to salivary glands (Su *et al.*, 2015).

Wolbachia is the most abundant endosymbiotic bacterium among arthropods, and therefore probably the most abundant intracellular symbiont on earth (Hilgenboecker *et al.*, 2008; Zug & Hammerstein, 2012; Zug & Hammerstein, 2015; Martinez *et al.*, 2016). It is vertically transmitted by females via the cytoplasm of the egg (Werren, 1997) and is often a manipulator of host reproduction, causing cytoplasmic incompatibility, thelytokous parthenogenesis, feminization or male killing (Werren *et al.*, 2008). It can also modify various life history traits of its host - positively or negatively - without causing

the above phenotypes (Vavre *et al.*, 1999; Stouthamer & Luck, 1993; Hosokawa *et al.*, 2010; Zug & Hammerstein, 2015; Martinez *et al.*, 2016; Lopez *et al.*, 2018). Its role in plant defense manipulation has been rarely studied but is gaining more and more attention. One of the first report of a potential link between *Wolbachia* and an insect host plant documented that the activation of defence-related genes in the plant was lower when fed on by *Wolbachia*-infected beetles (*Diabrotica virgifera virgifera*) (Barr *et al.*, 2010). Another pioneering study showed that *Wolbachia* infecting the leaf-mining moth *Phyllonorycter blancardella* (Lepidoptera) was involved in the production of cytokinins, plant hormones that inhibit senescence, maintain chlorophyll, and control nutrient mobilization in plant leaves. This prevents the local area of the leaves in which the insect feeds from senescing, leading to conspicuous "green islands" that remain photosynthetically active for the benefit of the leaf-mining host (Giron *et al.*, 2007; Kaiser *et al.*, 2010).

The cabbage root fly *Delia radicum* is an oligophagous insect specialist of the Brassicaceae family. It is an economically important pest of brassicaceous crops within the temperate zone of the holarctic region (Finch, 1989). This plant family is known to produce glucosinolates (GSLs), specialized compounds found in seeds, roots, stems and leaves and generally considered as important in its defense strategy against insects (Hopkins *et al.,* 2009). Intact GSLs are stored in the vacuoles of plant cells and are not toxic as such (van Dam *et al.*, 2009). However, upon tissue damage and cell rupture (e.g. herbivory), they are released and mixed with the enzyme myrosinase, which converts them into various toxic degradation products such as volatile isothiocyanates (ITCs) (Vig *et al.*, 2009). ITCs will attract and stimulate specialists, which can prevent their formation, but will on the contrary act as deterrents for generalist insects, which are unable to do so (Agrawal & Kurashige, 2003; Hopkins *et al.,* 2009; Ali & Agrawal, 2012). However, these volatile ITCs emissions can also indirectly affect specialists since they can also attract their natural enemies (Lamb, 1989; Bartlet, 1996; Murchie *et al.*, 1997). Root-feeding by *D. radicum* induces notable changes in the levels of GSLs both locally (in roots) and systemically (in leaves) (van Dam & Raaijmakers, 2006). This feeding also modifies the volatile blends emitted by the plant, which attract specialized natural enemies such as the parasitoid rove beetles *Aleochara bilineata*, *Aleochara bipustulata* and the parasitoid wasp *Trybliographa rapae* (Neveu *et al.*, 2002; Ferry *et al.*, 2007; Soler *et al.*, 2007) but also

conspecific and heterospecific competitors (Finch, 1978). For example, compounds such as dimethyl disulfide (DMDS) – a monomolecular volatile compound, frequent in sulfur-containing plants, and emitted in large amounts by brassica roots infested by *D. radicum* – plays a key role in the attraction of staphylinid predators and parasitoids (Ferry *et al.*, 2007; 2009) while ITCs are known to be attractive to the cabbage root fly itself (Finch & Skinner, 1982).

The microbiota of the cabbage root fly has been described recently (Bili *et al.*, 2016) and revealed a rich gut community (59-101 OTU depending on the population) as well as the presence of one endocellular symbiont: *Wolbachia sp*. While recent work (Lopez *et al.*, 2018) showed that this symbiont can influence important life history traits of *D. radicum* such as hatch rate, larvo-nymphal viability or development time, nothing is known about its potential influence on direct and indirect defenses of its host plant.

In this study, we investigated whether *Wolbachia* would modify the insect-plant dialogue in oilseed rape (*Brassica napus*) by comparing the plant defenses following phytophagy by *D. radicum* larvae infected or not by this intracellular symbiont. In particular, we measured and compared GSL compositions and volatile emissions in/from roots infested by *Wolbachia*-infected and *Wolbachia*-free larvae and compared them to those of healthy control plants.

MATERIAL & METHODS

Plant cultivation

Oilseed rape seeds (*Brassica napus L. subsp. oleifera cv. Tenor)* were germinated on glass beads in plastic food containers with transparent lids and kept for one week at constant temperature and humidity (24°C; 70% relative humidity (RH)) under long-day conditions (16 h : 8 h, light : dark cycle) in a climate chamber. The seedlings were then transferred to 2.2 L pots (11 x 11 x 21.5 cm) filled with potting soil covered with a thin layer of river sand. The seedlings were covered by small plastic cups to facilitate growth. Pots were enclosed in insect-proof nets to prevent infestation by thrips. Plants were watered with tap water as needed and grown in a greenhouse (Leipzig Botanical Garden, Germany) for five weeks before infestation.

Insect culture

The *D. radicum* strain used here was originally reared from 4000 pupae collected in 2014 in experimental broccoli fields at Le Rheu (Brittany, France, 48°07′16″N, 1°47′41″O). After emergence, these flies were reared in the lab in a climate-controlled room (21 ± 1°C, 60 ± 10% RH, L16:D8) on swede roots (*Brassica napus subsp. rapifera*) following a method derived from van Keymeulen *et al.* (1981). This lab stock was then used to create two separate sub-stocks bearing the same genetic background but a different infection status: a 100 % *Wolbachia*-infected line (WP i.e. "*Wolbachia*-plus") and a *Wolbachia*-free line (WM i.e. "*Wolbachia*-minus"), using isofemale lines (detailed protocol in Lopez *et al.*, 2018).

No specific permissions were required for this experiment which did not involve endangered or protected species.

Plant induction

After five weeks of growth, plants were infested by placing three L3 *D. radicum* larvae (12 days old) with a brush onto the soil surface, immediately adjacent to the stem of each plant. Plants were checked one hour later to control whether all larvae had disappeared into the sand. Ten replicates were used per infestation treatment (WP, WM), including ten more plants left uninfested as controls (CON).

Collection of root volatiles and GC-MS analysis

Roots volatiles emissions were sampled one day and three days after infestation. Based on previous studies with turnip (*Brassica rapa subsp. rapa*), this delay is sufficient to induce volatiles attracting parasitoids (Neveu *et al.*, 2002; Pierre *et al.*, 2011). On day 1 and 3, the base of each sampled plant was enclosed in a PET oven bag (Toppits © Bratschlauch) closed with a cable tie around the stem resulting in a semi-tight enclosure. The tie was made just below the stem of the first leaves so that the volatiles measured would come from roots (and soil) only. The insects remained in the plants during volatiles collection. A polyurethane (PUN) tube was inserted at the top of the bag and connected

to a vacuum pump system. Volatiles were collected on thermal desorption traps filled with 200mg Tenax®-GR Adsorbent Resin (Markes Environmental, USA). Nine plants from each treatment (WP and WM), five control plants (CON) and an air blank (to account for the volatiles naturally present in the greenhouse atmosphere in the analysis) were sampled simultaneously during 30 minutes (Figure 1). Collection flow rates were set to 0.2 L/min. After 30 minutes, the traps were removed from the pump, capped and kept at room temperature until analysis.



Figure 1. Experimental set-up for volatiles measurements.

The next day, volatiles were analyzed by a thermal desorption-gas chromatograph-mass spectrometer (TD-GC-MS) consisting of a thermodesoprtion unit (MARKES, Unity 2, Llantrisant, UK) equipped with an autosampler (MARKES, Ultra 50/50). Tubes were desorbed with helium as carrier gas and a flow path temperature of 150°C using the following conditions: dry purge 5 min at 20 ml/min, pre-purge 2 min at 20 ml/min, desorption 8 min at 280°C with 20 ml/min, pre-trap fire purge 1 min at 30 ml/min, trap heated to 300°C and hold for 4 min. The volatiles were separated on a gas chromatograph (Bruker, GC-456, Bremen, Germany) connected to a triple-quad mass spectrometer (Bruker, SCION). Separation took place on a DB-5MS column (30 m x 0.25 mm x 0.25 µm;

Restek, Germany). The oven was programmed from an initial temperature of 40°C (5 min hold), followed by an increase to 200°C at 20°C min^{-1} and hold for 5 min, then increased to 230°C at 30°C min^{-1}, and hold at 230 °C for 10 min. The MS was operated as follows: full scan from 33 to 500 m/z; electron energy, 70eV; transfer line temperature, 240°C; ion source temperature, 220°C; manifold 40°.

Root and leaf samples collection

Sampling of roots and leaves took place 7 days after infestation for the 30 plants (10 CON, 10 WM and 10 WP). For roots, the whole taproot with the surrounding small roots was sampled and rinsed with water. For leaves, the second newest leaf (counting the first as the first completely formed leaf) was cut and folded several times. All samples were wrapped in a 20 cm x 20 cm piece of aluminum foil, then immediately immersed in liquid nitrogen. They were then put for a few hours in a -80°C freezer, before being placed for 65 hours in a freeze dryer.

During plant collection, all roots were photographed to visually check for damage. All 20 plants infested with larvae were attacked, but damage levels varied. We scored them from 1 to 3 with 1: little damage, 2: approximately half of the root destroyed, 3: almost no root left. Overall, WP plants seemed visually a little more damaged than WM ones (Table 1).

Table 1. Damage levels on roots seven days after induction with WM and WP larvae.

Damage level	WM roots	WP roots
Little damage	6	3
≈ Half of root destroyed	3	6
Almost no root left	1	1

Glucosinolates extraction and HPLC analysis

GSLs were extracted according to Grosser & van Dam (2017). First, intact GSLs were extracted from 100 mg ground plant materials with a 70% methanol-water mixture at 90-92°C to disable myrosinase activity. Then, the resulting extract was brought into glass-columns containing dextran gel for purification. After adding sulfatase treatment, the columns were covered with aluminum foil and left overnight. Due to sulfatase activity, sulfate groups were removed, releasing the desulfoglucosinolates from the column so that we could elute them with 1.5 mL of ultrapure water. The samples were then freeze-dried. The residue was taken up in 1 mL of water, which was analyzed by high-pressure liquid chromatography (HPLC) with an ultraviolet (UV) detector. Detection and quantification were achieved by conducting comparisons with the retention times and UV spectra of commercial reference standards. The concentrations were calculated based on a sinigrin reference curve (for a detailed description, see Grosser & van Dam, 2017).

Statistical analysis

Pre-treatment of data

For the GSL dataset, some leaf samples were removed from the analysis (2 CON, 2 WM and 1 WP plants) because the plants were so small or damaged at the time of sampling that not enough leaf material was available for a successful extraction. Root and leaf GSLs were analyzed at the same time. This resulted in a dataset of 10 GSLs and 55 samples (roots: 10 CON, 10 WM and 10 WP; leaves: 8 CON, 8 WM and 9 WP).

For the two volatile datasets (day 1 and day 3), peaks relating to mixtures and impurities were removed. Then, the air blank average values were subtracted from the treatment values to account for the volatiles naturally present in the greenhouse. To avoid rare compounds that disproportionately could affect the outcome, only those compounds that were found at least five times in at least one treatment group were retained for the analyses. This resulted in a dataset of 15 volatiles for day 1 and 13 for day 3.

Statistics

Statistics were performed in the R.3.4.0 software (R Core Team, 2017), using the "vegan" (Oksanen *et al.*, 2017) and "RVAideMemoire" (Hervé, 2017) packages. All datasets were fourth-root transformed then centered and unit-variance scaled. Redundancy Analysis (RDA) and associated permutation tests were used to estimate the effect of experimental factors (Hervé *et al.*, 2018). The model applied on GSL data included as independent variables the plant organ, the treatment and the interaction between these two factors. Volatiles data were analyzed separately for each time point, with models that only included the treatment as independent variable. All datasets were also analyzed using univariate ANOVAs applied on each compound, with p-values adjusted using the False Discovery Rate correction for multiple testing (Benjamini & Hochberg, 1995).

RESULTS

Glucosinolates

Ten different GSLs were detected in our samples overall (see Table 2 for compound abbreviations used, Appendix 1). Figures 2a,b display the factorial map and the correlation circle of the RDA. This model explains 66% of the total variance and 95% of the constrained variance is explained by the first and the second components of the RDA (74.95% and 19.77% respectively). Figure 3 shows the mean concentrations of GSLs in leaves and roots and the differences between groups. Five GSLs are present in both organs while five others discriminate roots from leaves: RAPH, 4OH, ERU and NAS are only present in roots while 4MeOH is only found in leaves (Table 2; Figure 2a,b). GBC and NEO are also detected in much higher concentrations in leaves than roots (Figure 2a,b; Figure 3).

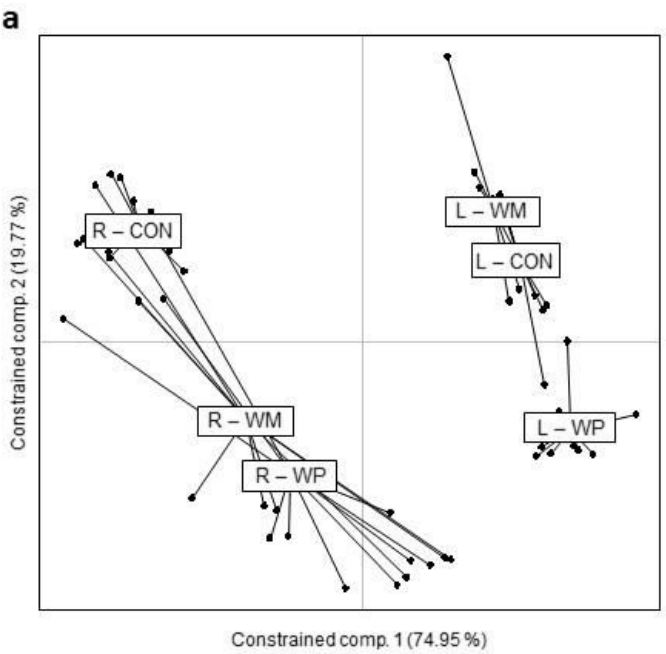
GSLs levels differ significantly between treatments but in a specific manner for each organ (Figure 2c). All combinations differ significantly from each other except WP roots *vs* WM roots and CON leaves *vs* WM leaves, which have similar GSLs contents (Figure 2d).

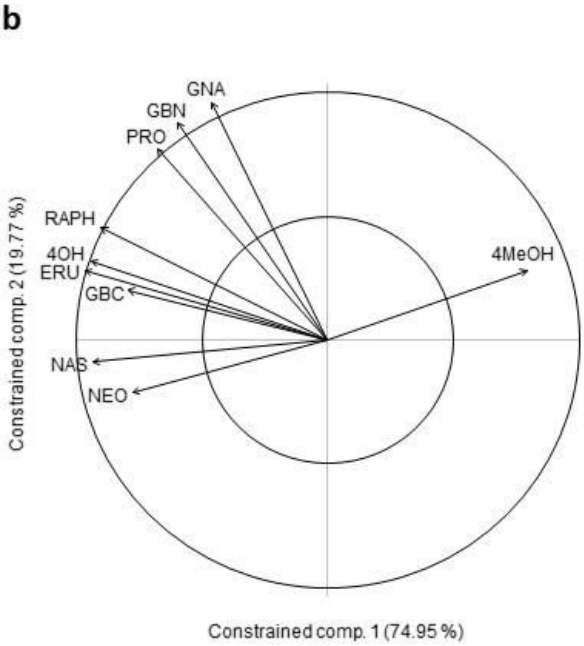
Table 2. Class, common names and abbreviations used for glucosinolates, and their location in the plants analyzed.

Class	Compound	Abbreviation used	Location
Indole	4-methoxyglucobrassicin	4MeOH	Leaves
	4-hydroxyglucobrassicin	4OH	Roots
	Neoglucobrassicin	NEO	Leaves & Roots
	Glucobrassicin	GBC	Leaves & Roots
Aliphatic	Glucoraphanin	RAPH	Roots
	Glucoerucin	ERU	Roots
	Progoitrin	PRO	Leaves & Roots
	Gluconapin	GNA	Leaves & Roots
	Glucobrassicanapin	GBN	Leaves & Roots
Aromatic	Gluconasturtiin	NAS	Roots

Roots

Root feeding by *D. radicum* larvae significantly alters the GSLs blend irrespective of the presence of *Wolbachia*, with an increase of NEO (indole), and a decrease in 4-OH (indole) and all aliphatic compounds (GNA, GBN, PRO, RAPH, and ERU) compared to control; (Figures 2b; 3). Although no significant difference between WP and WM treatments is found for any compound (Figures 2d; 3), observed concentrations are always lower in attacked plants and always the lowest in WP plants, with the exception of NEO, which is detected in significantly higher quantity in the WP treatment than the control one. Accordingly, the WP treatment differs significantly from CON in 7 cases (4OH, RAPH, ERU, NEO, PRO, GNA and CAN) but only in 3 cases for WM (4OH, RAPH and GNA) (Figure 3).





c

	F	df	*P*
Organ	63.86	1, 49	**< 0.001**
Treatment	9.61	2, 49	**< 0.001**
Organ x Treatment	4.86	2, 49	**< 0.01**

999 permutations

d

	L – CON	L – WM	L – WP	R – CON	R – WM
L – WM	0.193	-	-	-	-
L – WP	**< 0.01**	**< 0.01**	-	-	-
R – CON	**< 0.01**	**< 0.01**	**< 0.01**	-	-
R – WM	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	-
R – WP	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	0.418

L: leaf; R: root
999 permutations
P-value adjustement method: fdr

Figure 2. Statistical outputs for glucosinolates measured in leaves (L) and roots (R). CON: control; WM: *Wolbachia*-free; WP: infected with *Wolbachia*. Redundancy Analysis (RDA) (a) factorial map and (b) correlation circle. Permutation *F*-tests (c) of the factors included in the Redundancy Analysis (RDA) (999 permutations) and (d) to assess pairwise comparisons for organ and treatment. Significant *P*-values are **in bold**. 4MeOH: 4-methoxyglucobrassicin; 4OH: 4-hydroxyglucobrassicin; NEO: neoglucobrassicin; GBC: glucobrassicin, RAPH: glucoraphanin; ERU: glucoerucin; PRO: progoitrin; GNA: gluconapin; GBN: glucobrassicanapin; NAS: gluconasturtiin.

Leaves

In leaves, only the WP treatment differs significantly from control (Figure 2a,d). Therefore, plants react to a root attack by modifying their leaves GSLs blend but only when the attacking larvae are infected by *Wolbachia*. In WP leaves, concentrations seem particularly low for some aliphatic compounds (PRO, GNA and GBN) compared to control

and WM ones (Figure 3). However, as these compound-specific differences are not significant, we can say that the global difference between CON and WP, found by the RDA analysis, comes from the combination of lower levels of PRO, GBN and GNA in WP plants (Figure 3). Moreover, 4-MeOH (indole) is significantly *more* concentrated in WM leaves than in control or WP treatments (Figure 3).

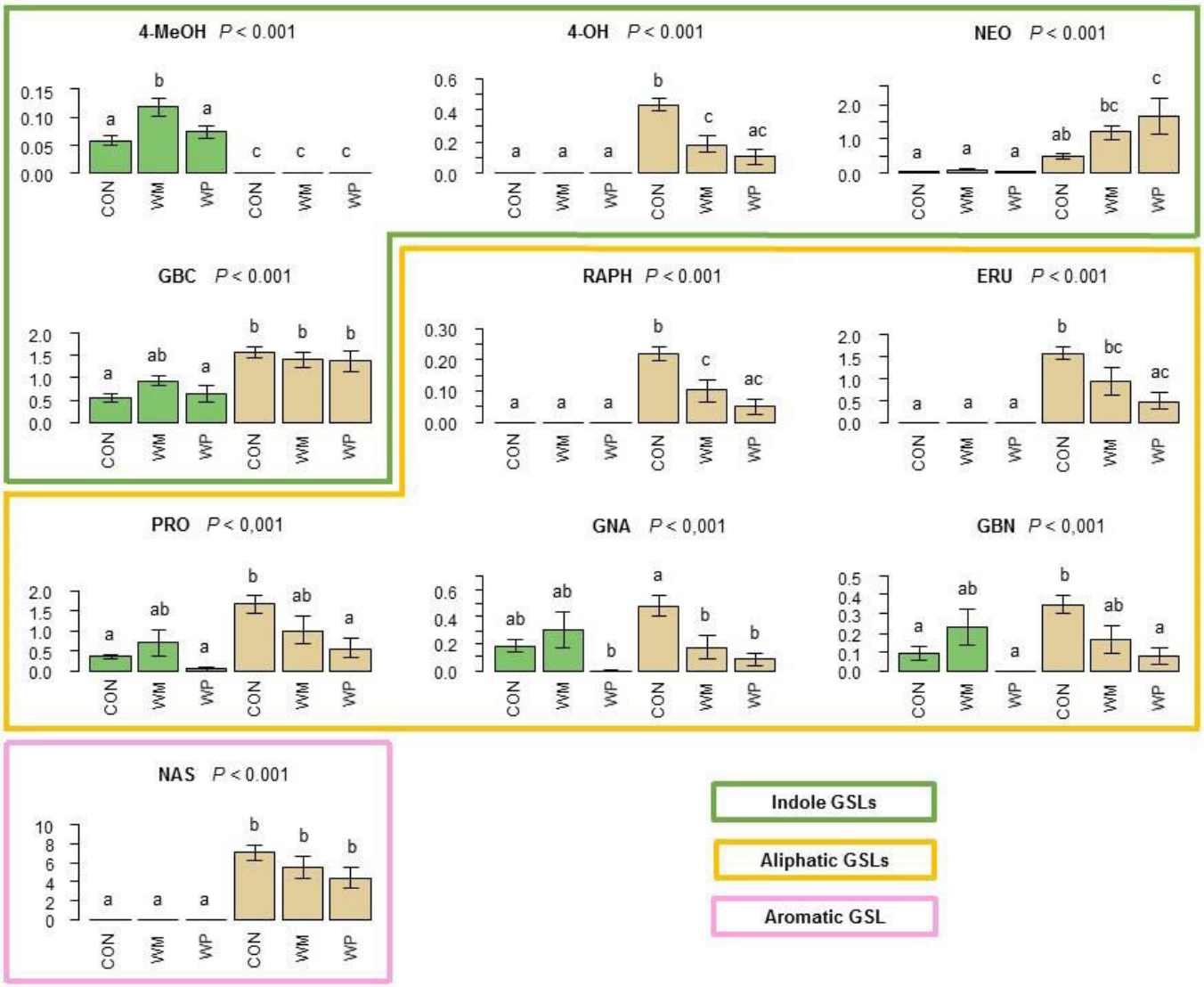


Figure 3. Mean concentrations of glucosinolates in leaves (green) and roots (light brown). CON: control; WM: *Wolbachia*-free; WP: infected with *Wolbachia*. Bars represent standard errors and different letters indicate statistically different groups. 4MeOH: 4-methoxyglucobrassicin; 4OH: 4-hydroxyglucobrassicin; NEO: neoglucobrassicin; GBC: glucobrassicin, RAPH: glucoraphanin; ERU: glucoerucin; PRO: progoitrin; GNA: gluconapin; GBN: glucobrassicanapin; NAS: gluconasturtiin.

Volatiles

Fifteen different volatiles were detected in our samples on day 1 (Appendix 2) and 13 on day 3 (Appendix 3). The RDA and associated permutation tests are displayed on Figures 4 and 5. On day 1, the model explains 14% of the total variance. All (100%) of the constrained variance is explained by the first and the second component of the RDA (88.83% and 11.17% respectively). On day 3, the model explains 10% of the total variance and again, 100% of the constrained variance is explained by the first and the second component of the RDA (81.6% and 18.4% respectively). No significant difference among treatments is found, neither for day 1 (Permutation F-tests, 999 permutations, F = 1.62, df = 2.2, P = 0.103) nor day 3 (Permutation F-tests, 999 permutations, F = 1.12, df = 2.2, P = 0.318).

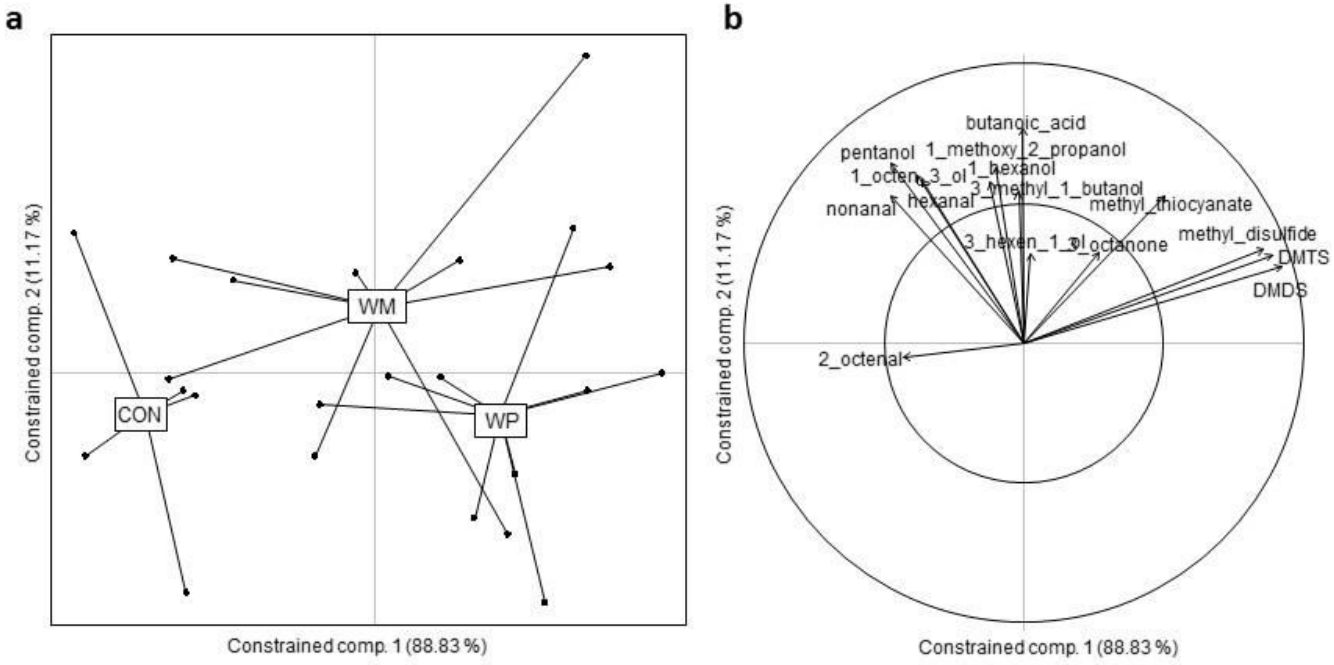


Figure 4. Statistical outputs for volatiles measured on Day 1. CON: control; WM: *Wolbachia*-free; WP: infected with *Wolbachia*. Redundancy Analysis (RDA) (a) factorial map and (b) correlation circle.

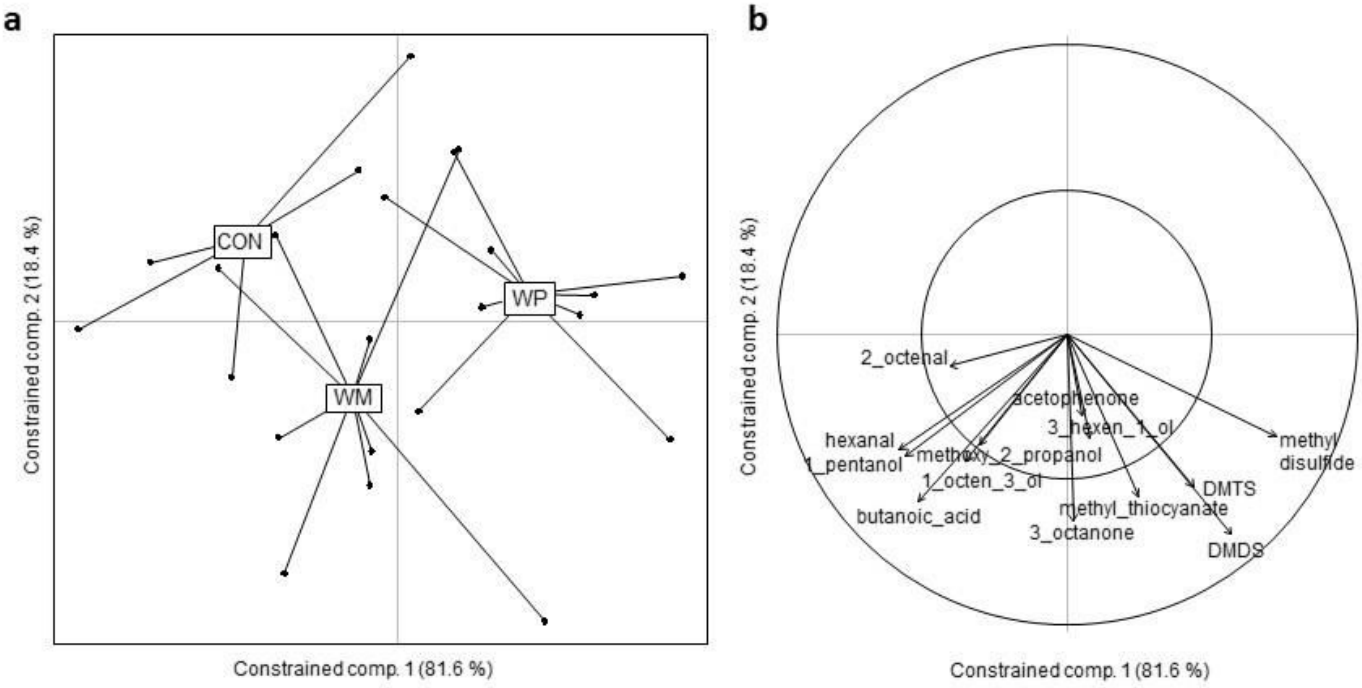


Figure 5. Statistical outputs for volatiles measured on Day 3. CON: control; WM: Wolbachia-free; WP: infected with Wolbachia. Redundancy Analysis (RDA) (a) factorial map and (b) correlation circle.

DISCUSSION

Our main finding is that the endocellular symbiont of a root herbivore can modulate the systemic production of plant defense compounds following an attack by the herbivore, as demonstrated by a significant modification of the foliar GSL profile which is restricted to the case when the root pest bears the symbiont. We also illustrate the qualitative and quantitative modifications in GSL profiles of the root itself following the attack, which are significant but do not appear related to the infection status of the herbivore. However, we do not detect modifications of the volatiles emitted by the roots attacked by *D. radicum* larvae after one or three days.

Wolbachia modifies glucosinolate levels in the leaf but not in the root

In this study, *Wolbachia*-infected larvae attacking roots specifically reduced GSLs concentrations *in the leaves* while herbivory by uninfected larvae had no detectable effect above ground. This was true especially for three aliphatic compounds (GBN, PRO, GNA),

where concentrations fell to undetectable levels (0.05 for PRO) for the WP treatment. The modification of GSLs concentrations in the leaves after root herbivory has already been observed in *Brassica* species (van Dam & Raaijmakers, 2006). Indeed, the presence of herbivores on one part of the plant is often a reliable indication that other parts of the plant will be attacked as well in due course.

We did not detect any influence of the *Wolbachia* infection on the GSLs produced in the roots being attacked. Since the symbiont has a detectable influence in the GSLs produced in the leaves, i.e. further from the site of attack, this result is surprising since one would expect the symbiont to be even more "useful" for its host at the direct location of feeding. However, as specialist herbivores such as *D. radicum* are able to detoxify or even prevent the formation of ITCs "on their own" when feeding (Agrawal & Kurashige, 2003; Hopkins *et al.,* 2009; Ali & Agrawal, 2012), we can imagine that *Wolbachia* does not really have a role to play here, since *D. radicum* is able to circumvent the effects of GSLs anyway.

Overall, GSL concentrations in the roots fell significantly in both treatments (WM and WP) following the attack. This may seem surprising as phytophagy usually leads to an increase in GSLs, as reviewed by Textor & Gershenzon (2009). However, these authors also noticed that plants have a tendency to decrease their levels of aliphatic GSLs in response to specialist herbivores. Indeed, two studies that measured GSLs levels in *Brassica* roots after the specialists *D. floralis* and *D. radicum* attacked the plants, showed up to 60% declines of aliphatic and aromatic GSLs, while indolic GSLs increased (Birch *et al.*, 1992; Hopkins *et al.*, 1998).

The fact that *Wolbachia*-infected larvae modify the above-ground defense reaction of the plant differently from uninfected ones is remarkable because it demonstrates that an intracellular symbiont can somehow influence the interaction between a pest and its host plant. This is consistent with another study that showed that plant defenses were decreased when attacked by *Wolbachia*-infected beetles (*Diabrotica virgifera virgifera*) compared to uninfected ones (Barr *et al.*, 2010).

When *D. radicum* larvae chew on *Brassica* tissues, GSLs get converted into toxic degradation compounds such as isothiocyanates (ITCs), which production will usually increase following an attack (Ratzka *et al.*, 2002). From an evolutionary point of view, it is therefore in the larvae's interest to minimize this defense reaction if possible (i) in the

roots, where the larvae are feeding and developing, because they will have to detoxify these defense compounds (Vig *et al.*, 2009); but also (ii) in the whole plant (roots *and* leaves), to prevent ITC volatile emissions that could attract their natural enemies or competitors (Lamb, 1989; Bartlet, 1996; Murchie *et al.*, 1997). Since *Wolbachia* are only maternally transmitted via the cytoplasm of the egg (Werren, 1997), they share the same interest as their host in a reduced plant defense, which favours *D. radicum* reproduction. Accordingly, any direct or indirect role they might play in reducing plant defense would be selected for. The symbiont could thus bring an advantage to its larval host by decreasing plant defenses above ground, and thus decreasing potential olfactive cues for its natural enemies. Moreover, because leaf GSLs are also used as oviposition stimulants for *D. radicum* (Finch & Skinner, 1982), it is also possible that lower GSLs concentrations in leaves could reduce intraspecific competition.

While the adaptive significance of our results still remains to be tested, they need to be taken with caution as the intensity of feeding could not be controlled in our experiment. Indeed, the difference between WP and WM treatments could also be due to small differences in the attack levels of the roots as we observed that WP roots were slightly more damaged than the WM ones.

How could an intracellular symbiont exert an influence outside its host?

In this study, *Wolbachia* seems able to alter the physiological reaction of a plant although it remains within the cells of its insect host, which begs the question of a likely mechanism linking the inside of the host's cells with the tissues of the plant. However, insects developing on plants deposit an abundance of cues that plants may perceive, and among these cues, compounds present in oral secretions or feces of herbivorous insects are frequently involved (Howe & Jander, 2008).

Interestingly, such cues could realistically be altered by a *Wolbachia* infection. Indeed, recent studies demonstrated that *Wolbachia* could shape the microbiota of its hosts. This was shown in another diptera, *Drosophila melanogaster* (Simhadri *et al.*, 2017; Ye *et al.*, 2017), and in the isopod *Armadillidium vulgare* (Dittmer & Bouchon, 2018). Particularly, Ye *et al.* (2017) showed that *Wolbachia* could significantly reduce its host gut microbiota diversity. It is thus possible that *Wolbachia* could influence indirectly the reaction of

plants to herbivory through a modification of the gut microbiota of their insect host, which could influence the composition of its feces or its oral secretions. A direct demonstration of *Wolbachia* altering the microbiota of *D. radicum* could be achieved by comparative 16S rDNA gene sequencing of microbiota in *Wolbachia*-infected and *Wolbachia*-free individuals.

Another non exclusive hypothesis would be that *Wolbachia* directly influences the insect saliva composition *in situ*, as these symbionts have repeatedly been found in the cells of insect saliva glands (Wolstenholme, 1965; Amuzu *et al.*, 2015). A logical complementary experiment would therefore be to screen *Wolbachia* in live insect tissues of our WP strain to detect whether or not it is present in the salivary glands of *D. radicum.* Through the use of a fluorescent dye called Syto-11, *Wolbachia* can be efficiently detected in various insect tissues. Due to background fluorescence, live imaging of Syto-11-stained *Wolbachia* cells requires confocal microscopy. The advantage of this staining approach is that it informs about *Wolbachia* titer as well as its tissue and cellular distribution (Casper-Lindley *et al.,* 2011).

Wolbachia does not influence volatiles emitted by infested roots… but neither does infestation

Wolbachia infection did not modify volatile blends emitted by the roots, but what is even more surprising is that infestation on its own did not have any effect either. Yet, in another study on *D. radicum* feeding on a closely related species, turnip (*B. rapa subsp. rapa*), infested roots were shown to emit volatiles that attracted a specialized parasitoid, *Trybliographa rapae* (Neveu *et al.*, 2002). Several issues could explain this discrepancy. First, we used plants potted in horticultural soil, which mimics a natural setting better than mineral sand or vermiculite but harbors an extremely rich bacterial and fungal community, which itself releases numerous volatile substances (Insam & Seewald, 2010; Schmidt *et al.*, 2016). Although we did our best to eliminate from our analysis the substances which were of a known fungal (or insect) origin for example, the background noise of soil volatiles might have swamped the plant root signal we tried to detect. Secondly, volatile emissions can be strongly influenced by the plant species (Paré & Tumlinson, 1999), duration of infestation and herbivores density (van Dam & Raaijmakers, 2006; Soler *et al.*, 2007; Bruinsma *et al.*, 2009; Cai *et al.*, 2014). In our

experiment, all these parameters were different than in the study of Neveu *et al.* (2002). Finally, we used a very limited sample here (5-9 plants per treatment), which may not have supplied sufficient power as far as root volatiles are concerned. We observe for example that sulfides (methyl disulfide, DMDS and DMTS) and one product of GSL degradation (methyl thiocyanate) seem to be emitted in larger quantities in the plants with larvae than in the control ones, but this difference is not significant.

As *Brassica* plants also emit above-ground volatiles when attacked, and as these leaf volatiles play a very important role in the recruitment of the parasitoids of *D. radicum* (Neveu *et al.*, 2002; Pierre *et al.*, 2011), it would be pertinent to measure volatiles emitted by leaves in further studies to unravel the possible effect of *Wolbachia* on indirect defenses. As the presence of the symbiont strongly decreases the GSLs concentrations in the leaves, it is reasonable to hypothesize that it could also reduce their volatile emissions, for the benefit of its insect host.

CONCLUSION

Our study shows that *Wolbachia,* an endocellular symbiont of a root herbivore, can influence a plant defense reaction above ground. These results confirm the potential role of this symbiont in host-plant relationships, and since *Wolbachia* is by far the most widespread endocellular symbionts in insects – having reached the status of obligate symbiont in some species (Hosokawa *et al.*, 2010) – considering it in further insect-plant studies is of major importance. While the adaptive significance of the physiological effects we found still remains to be explored in our host plant complex, we highlight the necessity of taking endocellular symbionts (in general) into account when studying the evolution of plant insect interactions. This already rich field of study will therefore become even richer.

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APPENDIXES

Appendix 1. Mean levels of glucosinolates (µmol/mg) (±SE) in leaves and roots of oilseed rape samples following an attack by larvae of *Delia radicum* infected (WP) or not (WM) by *Wolbachia*. CON: control plants (no attack). *F* = statistic value, df = degrees of freedom, *P* = p-value (in bold when significant). Glucosinolates are ordered according to their class: indole, aliphatic or aromatic. 4MeOH: 4-methoxyglucobrassicin; 4OH: 4-hydroxyglucobrassicin; NEO: neoglucobrassicin; GBC: glucobrassicin, RAPH: glucoraphanin; ERU: glucoerucin; PRO: progoitrin; GNA: gluconapin; GBN: glucobrassicanapin; NAS: gluconasturtiin.

Class *Compound*				Treatment					
				Leaf			Root		
	F	df	*P*	CON	WM	WP	CON	WM	WP
Indole									
4MeOH	36.40	2	**< 0.001**	0.059 (0.008)	0.118 (0.017)	0.074 (0.012)	0 (-)	0 (-)	0 (-)
4OH	22.48	2	**< 0.001**	0 (-)	0 (-)	0 (-)	0.44 (0.04)	0.184 (0.057)	0.102 (0.043)
NEO	6.94	2	**< 0.001**	0.039 (0.006)	0.084 (0.012)	0.037 (0.012)	0.477 (0.085)	1.185 (0.216)	1.639 (0.537)
GBC	6.71	2	**< 0.001**	0.536 (0.097)	0.921 (0.124)	0.642 (0.168)	1.572 (0.115)	1.388 (0.177)	1.355 0.232
Aliphatic									
RAPH	16.18	2	**< 0.001**	0 (-)	0 (-)	0 (-)	0.22 (0.02)	0.102 (0.036)	0.049 (0.025)
ERU	13.38	2	**< 0.001**	0 (-)	0 (-)	0 (-)	1.573 (0.148)	0.923 (0.319)	0.478 (0.187)
PRO	6.08	2	**< 0.001**	0.34 (0.06)	0.704 (0.309)	0.048 (0.048)	1.658 (0.207)	1.005 (0.333)	0.556 (0.228)
GNA	5.19	2	**< 0.001**	0.184 (0.043)	0.304 (0.137)	0.003 (0.003)	0.479 (0.076)	1.171 (0.088)	0.081 (0.047)
GBN	4.93	2	**< 0.001**	0.093 (0.038)	0.23 (0.09)	0 (-)	0.35 (0.04)	0.164 (0.072)	0.079 0.045)
Aromatic									
NAS	16.47	2	**< 0.001**	0 (-)	0 (-)	0 (-)	7.063 (0.816)	5.531 (1.173)	4.36 (1.04)

Appendix 2. Peak areas of volatile compounds (±SE) emitted by the roots of oilseed rape samples for **Day 1**. *F* = statistic value, df = degree of freedom, *P* = p-value. CON: control; WM: *Wolbachia*-free; WP: infected with *Wolbachia*.

Class *Compound*	*F*	df	*P*	Treatment		
				CON	WM	WP
Alcohol						
3-methyl-1-butanol	1.55	2	0.49	$0.3 .10^6$ ($0.1 .10^6$)	$0.6 .10^6$ ($0.2 .10^6$)	$0.3 .10^6$ ($0.1 .10^6$)
1-hexanol	1.80	2	0.49	$1.9 .10^6$ ($1.3 .10^6$)	$0.8 .10^6$ ($0.2 .10^6$)	$0.5 .10^6$ ($0.1 .10^6$)
1-octen-3-ol	1.52	2	0.49	$4.9 .10^6$ ($2.8 .10^6$)	$2.8 .10^6$ ($0.5 .10^6$)	$2.1 .10^6$ ($0.3 .10^6$)
1-pentanol	2.04	2	0.49	$0.9 .10^6$ ($0.5 .10^6$)	$0.6 .10^6$ ($0.1 .10^6$)	$0.3 .10^6$ ($0.1 .10^6$)
1-methoxy-2-propanol	0.05	2	0.96	$13.7 .10^6$ ($7.3 .10^6$)	$13.1 .10^6$ ($3.7 .10^6$)	$15.5 .10^6$ ($7.6 .10^6$)
3-hexen-1-ol	0.47	2	0.67	$0.8 .10^6$ ($0.4 .10^6$)	$0.3 .10^6$ ($0.1 .10^6$)	$0.7 .10^6$ ($0.5 .10^6$)
Aldehyde						
2-octenal	0.65	2	0.66	$0.3 .10^6$ ($0.2 .10^6$)	$0.1 .10^6$ ($0.1 .10^6$)	$0.2 .10^6$ ($0.1 .10^6$)
hexanal	0.93	2	0.62	$4.3 .10^6$ ($2.8 .10^6$)	$3.6 .10^6$ ($1.2 .10^6$)	$1.74 .10^6$ ($0.4 .10^6$)
nonanal	1.44	2	0.49	$3.9 .10^6$ ($1.3 .10^6$)	$2.8 .10^6$ ($1.2 .10^6$)	$0.9 .10^6$ ($0.6 .10^6$)
Acid						
butanoic acid	0.67	2	0.66	$0.2 .10^6$ ($0.1 .10^6$)	$0.4 .10^6$ ($0.1 .10^6$)	$0.3 .10^6$ ($0.1 .10^6$)
Ketone						
3-octanone	0.53	2	0.67	$0.3 .10^6$ ($0.2 .10^6$)	$0.2 .10^6$ ($0.1 .10^6$)	$0.2 .10^6$ ($0.1 .10^6$)
acetophenone	-	-	-	-	-	-
Sulfide						
methyl disulfide	2.67	2	0.49	$0.1 .10^6$ ($0.1 .10^6$)	$0.5 .10^6$ ($0.2 .10^6$)	$1.1 .10^6$ ($0.4 .10^6$)
dimethyldisulfide	2.58	2	0.49	$17.5 .10^6$ ($15.4 .10^6$)	$998.4 .10^6$ ($360.6 .10^6$)	$1256.6 .10^6$ ($381.3 .10^6$)
dimethyltrisulfide	1.31	2	0.49	$0.3 .10^6$ ($0.1 .10^6$)	$2.5 .10^6$ ($0.9 .10^6$)	$4.4 .10^6$ ($2.2 .10^6$)
Isothiocyanate						
methyl thiocyanate	1.40	2	0.49	$0.1 .10^6$ ($0.1 .10^6$)	$0.2 .10^6$ ($0.1 .10^6$)	$0.3 .10^6$ ($0.1 .10^6$)

Appendix 3. Peak areas of volatile compounds (±SE) emitted by the roots of oilseed rape samples for **Day 3**. *F* = statistic value, df = degree of freedom, *P* = p-value. CON: control; WM: *Wolbachia*-free; WP: infected with *Wolbachia*.

Class *Compound*	*F*	df	*P*	Treatment		
				CON	WM	WP
Alcohol						
3-methyl-1-butanol	-	-	-	-	-	-
1-hexanol	-	-	-	-	-	-
1-octen-3-ol	1.55	2	0.53	$2.7 .10^6$ ($0.9 .10^6$)	$2.8 .10^6$ ($0.5 .10^6$)	$1.6 .10^6$ ($0.4 .10^6$)
1-pentanol	1.63	2	0.53	$0.5 .10^6$ ($0.3 .10^6$)	$0.3 .10^6$ ($0.1 .10^6$)	$0.2 .10^6$ ($0.1 .10^6$)
1-methoxy-2-propanol	2.37	2	0.53	$9.3 .10^6$ ($3.2 .10^6$)	$6.8 .10^6$ ($1.2 .10^6$)	$4.1 .10^6$ ($1.1 .10^6$)
3-hexen-1-ol	1.18	2	0.53	$0.2 .10^6$ ($0.1 .10^6$)	$1.1 .10^6$ ($0.6 .10^6$)	$0.4 .10^6$ ($0.2 .10^6$)
Aldehyde						
2-octenal	1.61	2	0.53	$0.3 .10^6$ ($0.1 .10^6$)	$0.1 .10^6$ ($0.1 .10^6$)	$0.1 .10^6$ ($0.1 .10^6$)
hexanal	1.11	2	0.57	$2.7 .10^6$ ($2.1 .10^6$)	$1.5 .10^6$ ($0.7 .10^6$)	$0.6 .10^6$ ($0.3 .10^6$)
nonanal	-	-	-	-	-	-
Acid						
butanoic acid	1.51	2	0.53	$0.2 .10^6$ ($0.1 .10^6$)	$0.4 .10^6$ ($0.2 .10^6$)	$0.1 .10^6$ ($0.1 .10^6$)
Ketone						
3-octanone	0.40	2	0.78	$0.2 .10^6$ ($0.1 .10^6$)	$0.3 .10^6$ ($0.1 .10^6$)	$0.4 .10^6$ ($0.2 .10^6$)
acetophenone	0.33	2	0.78	$0.2 .10^6$ ($0.1 .10^6$)	$0.4 .10^6$ ($0.2 .10^6$)	$0.5 .10^6$ ($0.2 .10^6$)
Sulfide						
methyl disulfide	1.53	2	0.53	0 (-)	$0.1 .10^6$ ($0.1 .10^6$)	$0.1 .10^6$ ($0.1 .10^6$)
dimethyldisulfide	0.97	2	0.57	$2.6 .10^6$ ($0.6 .10^6$)	$149.9 .10^6$ ($87.6 .10^6$)	$122.7 .10^6$ ($53.1 .10^6$)
dimethyltrisulfide	0.79	2	0.61	$0.3 .10^6$ ($0.1 .10^6$)	$0.4 .10^6$ ($0.1 .10^6$)	$0.5 .10^6$ ($0.1 .10^6$)
Isothiocyanate						
methyl thiocyanate	0.19	2	0.83	$0.2 .10^6$ ($0.1 .10^6$)	$0.3 .10^6$ ($0.1 .10^6$)	$0.3 .10^6$ ($0.1 .10^6$)

GENERAL DISCUSSION

General discussion

One of the main objectives of this PhD was to unravel some of the tri-trophic interactions taking place between a host plant: the oilseed rape (*Brassica napus*), one of its phytophagous insect: the cabbage root fly (*Delia radicum*), and the microorganisms colonizing this fly, whether they live in the gut microbiota or within the cells. Indeed, although we have some knowledge about the interactions between *D. radicum* and its host plants, nothing has been published yet on the influence that insect symbionts can have on this fly, and even less on a higher trophic level, the plant it feeds on.

In this thesis, we started by studying the influence of the endocellular symbiont *Wolbachia* on the life history traits (LHT) of the cabbage root fly by comparing *Wolbachia*-infected and uninfected flies of the same population. Then, using a *Wolbachia*-free strain, we studied the role of the whole gut bacterial microbiota on these same LHT by comparing control flies with offspring of antibiotic-treated ones. In the last part of this thesis, we tested whether an insect *endocellular* symbiont such as *Wolbachia* could nevertheless influence the defense reaction of the plant to an attack by its insect host. More specifically, we tested if the chemistry of oilseed rape roots and leaves was modified differently following attacks by *Wolbachia*-infected or *Wolbachia*-uninfected *D. radicum* larvae.

Here we will discuss the main results we obtained, the limitations of our approaches and how some biases could be corrected in future experiments. To do so, conclusions from data presented in the manuscript will be completed with original data also collected during this PhD and with computer simulations using our own LHT estimates to model the frequency of a *Wolbachia* infection in a random-mating population.

Small and compensating effects of *Wolbachia* on "classical" life history traits...

Our results showed that *Wolbachia* infection had negative, neutral or positive effects depending on the LHT we measured (Figure 1). On the one hand, the symbiont was costly for starved *D. radicum* females when they laid eggs, since they suffered a 20% higher

mortality during a 3-day oviposition period compared to uninfected females. However, *Wolbachia* infection did not reduce viability in starving females which were not given the opportunity to lay eggs, suggesting that the energetic cost of the infection was only revealed in extreme conditions where unfed females had to exhaust their body reserves to produce eggs. Other negative effects of *Wolbachia* infection were a 10% lower hatch rate and a 1.5% longer development time from egg to adult. On the other hand, *Wolbachia* was also beneficial: it improved larvo-nymphal viability enough so that infected eggs, despite their reduced hatch rate, yielded as many adults as uninfected ones.

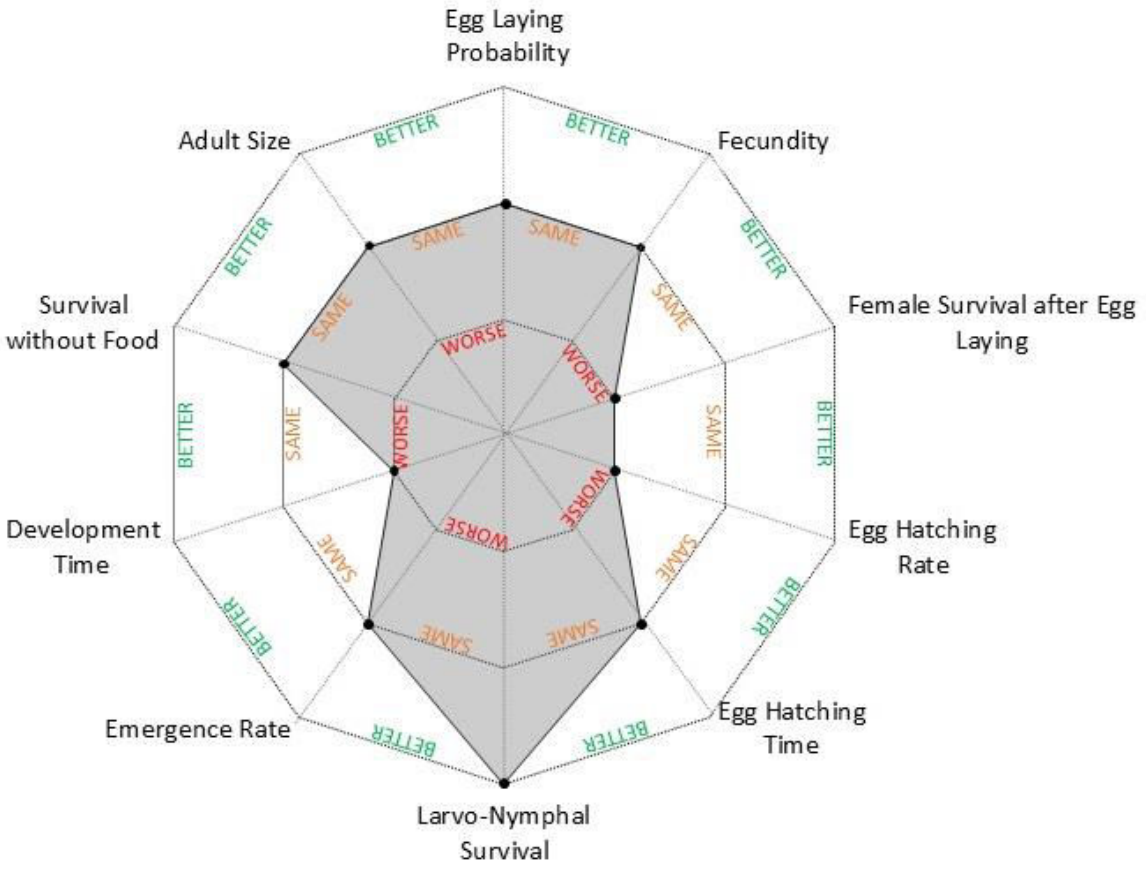


Figure 1. Radar chart illustrating the negative ("worse"), neutral ("same") and positive ("better") effects of *Wolbachia* on the ten life history traits of *Delia radicum* measured during this thesis. Specifically, "worse", "same" or "better" refer to the status of infected individuals relative to uninfected ones.

Overall, even if some of the effects we observed were statistically significant, they were all moderate (effect sizes in the 1-20% range). In addition, they seemed to mutually compensate each other, suggesting at first sight that *Wolbachia* infection might be nearly

benign in this host and might only drift slowly (but see our computer simulation and cage experiment further below).

Because *Wolbachia* is only transmitted vertically through the females, and thus, via host reproduction, the infection is not expected to be virulent since harming the host would reduce vertical transmission whereas a beneficial effect would enhance it. However, so far, all kinds of *Wolbachia* phenotypes have been observed, both positive and negative for the host, and bearing on very diverse traits. For example, the highly virulent *popcorn Wolbachia* variant infecting *Drosophila melanogaster* causes widespread degeneration of the brain, retina and muscles, leading to early death (Min & Benzer, 1997). On the contrary, *Wolbachia* can have highly positive effects such as increased female fecundity in the parasitoid wasp *Trichogramma bourarachae* (Vavre *et al.*, 1999), enhanced fertility in males of the stalk-eyed fly *Sphyracephala beccarii* (Hariri *et al.*, 1998), or protection against diverse RNA viruses in *D. melanogaster* (Hedges *et al.*, 2008). Mutually compensating effects such as those we observed in our study have been reported as well. In the flour beetle *Tribolium confusum* (Wade & Chang, 1995), *Wolbachia* infected females have fewer offspring than uninfected ones, but infected males are more fertile. These opposing effects indicate a much more complex relationship between the two organisms than a simple improvement/decline of some traits for the benefit/disadvantage of the host, and consequently, its symbiont.

… and a perfect transmission rate…

As it is only vertically transmitted, the success of *Wolbachia* is critically dependent on the efficiency of its maternal transmission. In the sample we tested, transmission rate was perfect, which is not an isolated case. This parameter has been measured many times in *Drosophila* species and in field populations: it averages 96% in *Drosophila melanogaster* and 95–97% in *Drosophila simulans*, with transmission efficiency of individual females varying from 60 to 100% (Kittayapong *et al.*, 2002). Because *recent* infections by symbionts are more likely to result in negative phenotypes and imperfect transmission due to a lack of coadaptation between the two partners (Turelli & Hoffmann, 1991; Turelli, 1994; Poinsot and Merçot, 1997), we suggest that *D. radicum* and *Wolbachia*

already have a long association history. This would also be consistent with the relatively modest effects of the infection we found on LHTs of its host.

During this PhD, our lab strains were regularly sampled (for various reasons) and tested to check their *Wolbachia* infection status (W+ or W-). This status was always assessed by PCR using primers FbpA_F1 and FbpA_R1. Within a year, the lab stock was checked four times on 64 to 316 individuals per assay, and the infection rate was comprised between 42% and 55% (Table 1). This is also consistent with our previous conclusions as we expect an old infection, with a low impact on LHTs, and a perfect vertical transmission, to be nearly benign in *D. radicum* and to drift slowly within populations.

Table 1. Percentage of infected individuals in our laboratory strain depending on the date of sampling. For each date, the number of individuals sampled is shown. Individuals were sampled randomly in the cages and the samples include males and females, aged 7-30 days. The reason for each PCR analysis is indicated.

Date of sampling	Number of individuals	Infected individuals	Reason
Oct. 2016	188	46 %	Evaluate the prevalence of *Wolbachia* in our lab strain
Feb. 2017	316	48 %	Attempt to create isofemale x isomale lines → failure, females refused to mate
Apr. 2017	87	55 %	Attempt to create isofemale lines (#1) → failure because some infected flies were detected in our presumably *Wolbachia*-free strain
Oct. 2017	78	42 %	Creation of isofemale lines (#2) → success! The W+ and W- lines obtained were the ones used for our life history traits experiment.

In any case, vertical transmission rate of *Wolbachia* is usually better in laboratory stocks than in the field, probably because of the controlled (and often ideal) conditions (Hoffmann *et al.*, 1990); it is therefore possible that the 100% transmission rate we observed in this study overestimates the situation in nature.

… yet a low prevalence in nature

To check prevalence in nature, some *D. radicum* wild populations were sampled in 2016 in four widely-spaced places in Brittany (one in each administrative department): Taulé (Finistère), Pleumeur (Côtes d'Armor), Pleugriffet (Morbihan) and Le Rheu (Ille-et-Vilaine).

To do so, we collected pupae in the field in all four places during the same period, then stored them in vermiculite in the laboratory until emergence. Pupae were checked twice a week and half of the emerging adults were put in a cage with food *ad libitum* and water while the other half was directly euthanized and kept in 96° alcohol. PCR were then performed on the "freshly" emerged adults and also on adults from the cages that had naturally died after several weeks.

Considering the high infection level of our laboratory stock, a very surprising result was the low prevalence of *Wolbachia* in wild populations, one of them (Pleumeur) even appearing *Wolbachia*-free, although our sample was limited (Figure 2). Among the other three other populations, the infection rates were low, ranging from 2 to 10% only. Pooling these data would provide an estimated 4% mean *Wolbachia* prevalence in Brittany (95%CI: 3-6%, N=508).

These results confirm that infection levels in the lab can largely differ from what is actually happening in nature. Our lab strain, infected at about 50% during most of my thesis, was originally sampled in Le Rheu in 2014, so the chances are that at the time of sampling, the prevalence was also lower than 10% for this strain. The perfect conditions in the lab probably allowed *Wolbachia* to proliferate rather quickly.

However, even considering a perfect vertical transmission, the symbiont can only invade a large cage population by providing a benefit of some kind to its host (otherwise, the population would just be perfectly maintaining its initial 10% infection rate). In our LHT experiment, we did not detect any obvious global positive effect that could explain the shift from an initial (supposed) 10% rate to the steady 50% of our laboratory stock. However, even though we are not able to explain an increase in the lab, we can hypothesize why there would be a decrease in nature. In realistic and more stressful conditions in the field, it is possible that carrying *Wolbachia* comes with a cost for *D.*

radicum, which could explain the low prevalence, or even the putative absence of the symbiont in one case.

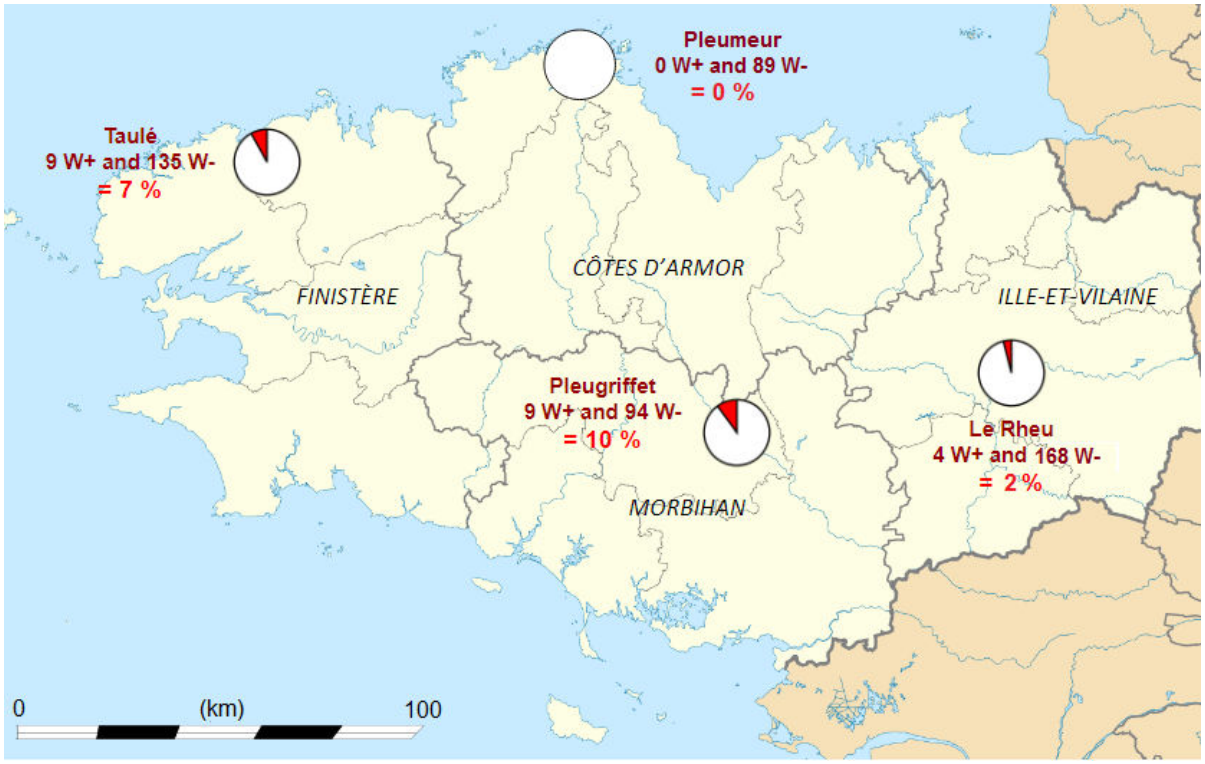


Figure 2. Percentage of *Wolbachia*-infected individuals in various wild populations of *Delia radicum* in Brittany. For each location, the numbers of *Wolbachia*-infected (W+) and -uninfected (W-) individuals in the sample are shown, with the corresponding percentage of infection. The samples include males and females.

Another explanation could be that in the field, several factors eliminate *Wolbachia* from the individuals during their lifetime. For example, flies could feed on substrates that might contain natural antibiotics. This is especially likely for larvae feeding in the rhizosphere, which is a place where living microorganism produce antibiotics as secondary metabolites (Geetanjali & Jain, 2016). In addition, flies could be subjected to climatic conditions/ thermal shocks in the wild that might eliminate their symbionts (as *Wolbachia* is known to be very sensitive to increases of temperatures) (Stevens, 1989).

Finally, we also suspect that during the PCR analysis of the wild populations, *Wolbachia* could only be detectable in half of the flies we used. Indeed, personal observations during the many PCRs we performed afterwards made us suspect that when the flies are sampled

right after their emergence, *Wolbachia* is not detectable yet. Indeed, we once sampled 300 individuals right after their emergence in our 50% infected-lab strain to check for their infection rate, and all the flies were identified as non-infected. Fifteen days later, we sampled again 300 adults about 10 days old, and the prevalence was "back to normal" with about 48% infected individuals. Therefore, the apparently very low prevalence observed in field individuals might not have been as low if the PCRs had been made only on individuals after they died naturally in the cages instead of doing half of them on newly emerged euthanized adults. When separating the data between both groups, this assumption seems to be confirmed, although the overall field prevalence does not seem to exceed 11% anyway (Table 2).

Table 2. Percentage of *Wolbachia*-infected individuals in four wild populations of *Delia radicum* in Brittany. For each location, the numbers of newly emerged and naturally dead individuals sampled are shown, with the corresponding percentage of infection. The samples include males and females. A fisher exact test pooling all data allows to conclude that infection is significantly more frequently found in naturally dead (i.e. old) individuals than in freshly emerged ones ($P = 0.013$).

	Newly emerged	Naturally dead
Taulé	3/82 = **3.6 %**	6/53 = **11.3 %**
Pleumeur	0/48 = **0 %**	0/41 = **0 %**
Pleugriffet	4/47 = **8.5 %**	5/47 = **10.6 %**
Le Rheu	0/93 = **0 %**	4/79 = **5.1 %**

A further step would be to expand this pupae field sampling to a larger area (France or even Europe), to determine if the Breton situation is representative of *Wolbachia* prevalence in *D. radicum* or peculiar. Based on our experience, we would advise that the adults emerging from the pupae be kept for at least 10 days before PCR typing. This complementary study would allow in particular to detect a possible geographic gradient in the prevalence of the infection. Indeed, a relationship has already been found between *Wolbachia* infections and latitudinal distribution. In the ants from the genus *Solenopsis* in Brazil, northern, central-western, and northeastern populations have low or no *Wolbachia* infection rates, indicating that incidence is apparently lower in regions with long dry seasons or high daily average temperatures (Martins *et al.*, 2012). On the

contrary, there was a higher *Wolbachia* prevalence occurring in more southerly temperate populations (Ahrens & Shoemaker, 2005). This makes sense since we know that *Wolbachia* is very sensitive to high temperatures (van Opijnen & Breeuwer, 1999).

Modeling of a *Wolbachia* infection reveals that, based on our LHT measurements, the symbiont should invade the populations of *Delia radicum*

In order to determine if the LHT measurements we had carried out were sufficient to justify the stability of the infection in our laboratory strain, we used them to build a simulation model. To do so, we adapted a model from Farkas *et al.* (2017) and simulated the evolution of the *Wolbachia*-infection rate in 100 independent virtual cage populations, assuming a perfect maternal vertical transmission, no cytoplasmic incompatibility, and the LHT values we had estimated in Chapter 1. To the original, deterministic model of Farkas *et al.* (2017), we added a stochastic component to simulate the behaviour of populations of laboratory-realistic size. The detailed model is presented in Appendix 1.

To extract the general trend shown by the 100 populations, we used a two steps process:

(i) for each generation, the mean infection rate of females was computed from the 100 populations simulated (→ 1 point/generation);

(ii) an asymptotic model was fitted on these mean values (constrained so that the asymptote was comprised between [0;1].

Contrary to our hypothesis of a slow drift, the model showed that, with these LHTs parameters, the *Wolbachia* infection should have invaded our laboratory strain and would go to near fixation in 50 generations (Figure 3). However, this is not what we had observed previously since the infection rate in our rearing facility had remained quite stable and near 50% during the course of a year (i.e. approximately 12 generations) before we built this simulation model (Table 1).

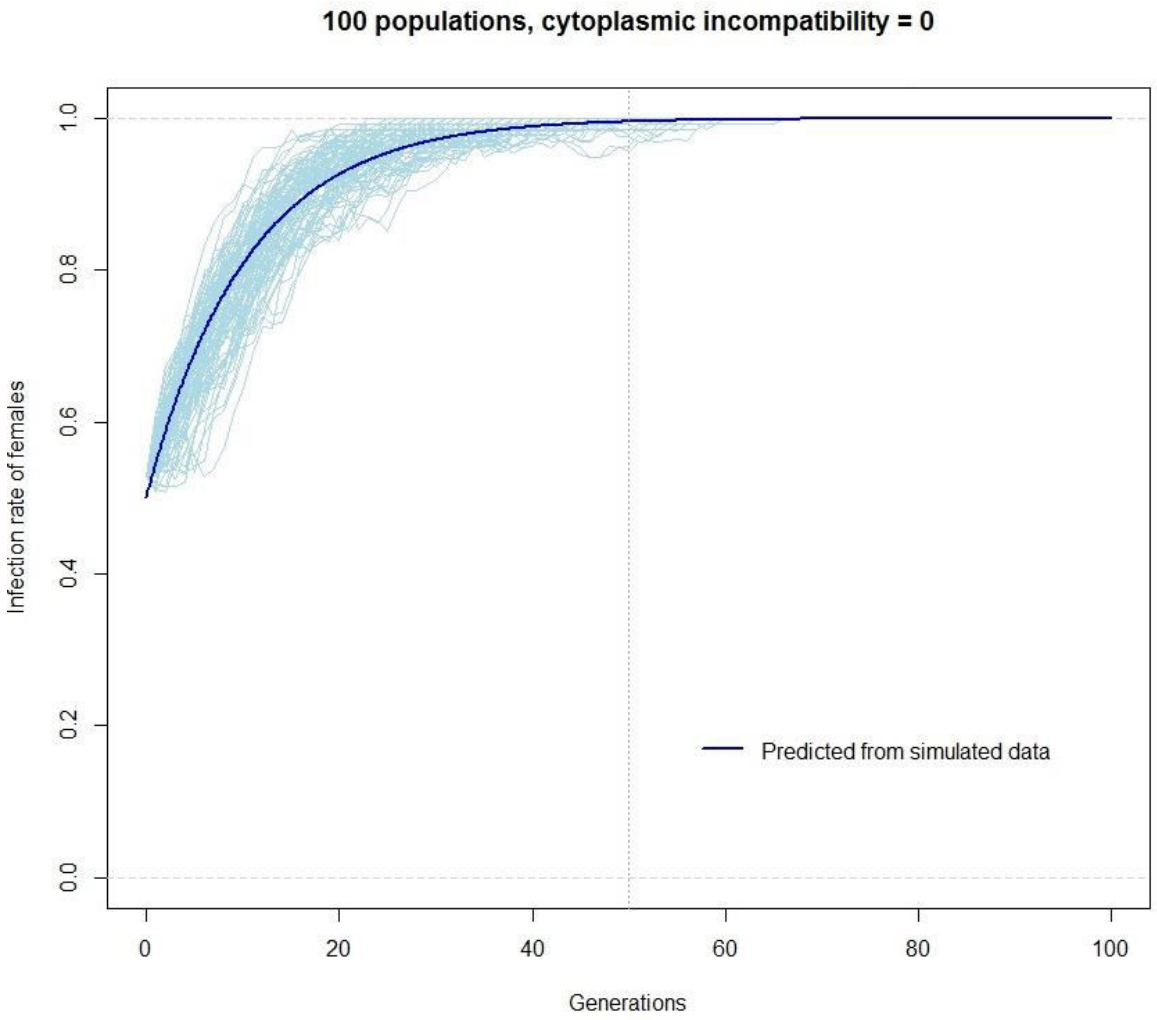


Figure 3. Predicted invasion dynamics of a *Wolbachia* infection in virtual *Delia radicum* cage populations under laboratory conditions, assuming perfect vertical transmission, no cytoplasmic incompatibility and life history trait estimated values as per Chapter 1. Thin clear blue lines represent the 100 populations simulated, and the dark blue line, the asymptotic model fitted on their average. The vertical grey dashed line shows the near fixation of the infection around generation 50.

Experiment to compare real cage data to the simulated model

Therefore, we ran a real cage experiment to track the infection rate of *Wolbachia* more precisely in our lab strain. In our stock, infected at 53% with *Wolbachia* at the beginning of this particular experiment, we randomly sampled 1080 adult flies (540 females and 540 males) aged 24–72 hours and randomly put them in three different cages (A, B and C), each containing 180 females and 180 males, to constitute generation 0 (G0). During

the whole experiment, which lasted 8 generations (i.e. approximately one year), the flies were fed *ad libitum* and given tap water.

When the flies were aged 12-18 days, a piece of swede root was introduced into each cage and left for 48 hours for the females to lay their eggs. All eggs were then collected and for each cage, 600 eggs were kept and deposited on two swede roots (300 eggs / root) for them to develop into adults that would constitute the next generation (G1). The adults from the previous generations were then euthanized. Amongst these dead flies, 100 females were kept individually in 96° alcohol to further check their infection status via PCR. This whole process was repeated for eight generations. However, due to time and budget constraints, PCRs were only made on the first two generations (G0 and G1), the "middle" one (G4) and the two last ones (G7 and G8). The percentages of infected females for each generation/cage are shown in Table 3.

Table 3. Estimated frequencies of *Wolbachia* infection in three independent *Delia radicum* real cage populations followed for eight non-overlapping generations of rearing in laboratory conditions. Each estimate is based on PCR carried out using 100 females per assay.

Generation	Cage A	Cage B	Cage C
G0	46 %	54 %	59 %
G1	48 %	68 %	58 %
G2	-	-	-
G3	-	-	-
G4	61 %	89 %	61 %
G5	-	-	-
G6	-	-	-
G7	78 %	93 %	89 %
G8	86 %	80 %	82 %

Wolbachia quickly invades the populations as predicted

Our PCR results showed that indeed, the *Wolbachia* infection rate did not remain stable but increased surprisingly rapidly in the three cages of our experiment (from 46% to 86%

in cage A, from 54% to 80% in cage B and from 59% to 82% in cage C). This result was strikingly different from the stability of the infection (approx. 50%) that we had observed previously in our routine lab rearing.

Our first thought was that this difference could be linked with the extra care that the three strains had received during the experiment. Indeed, cages were far less crowded than in the regular lab rearing, the wet cotton supplying water was changed and dead flies removed every other day, and the food was renewed every week, to keep the cages as clean as possible. Also, when preparing the eggs for the next generation, we made sure larvae would have more than enough resources to develop. For a good development, we usually consider that each larva needs 1g of root. In this cage experiment, and contrary to our routine rearing, eggs were counted individually and we used 50% larger roots than usual (e.g. 300 eggs were deposited on a 450g root).

On the contrary, in our large laboratory stock, where generations overlap since new individuals are added continuously, flies are more numerous in each cage, leading to a much higher density; food is never changed during the "cage life" (the stock is transferred to a new clean cage every about 30 days); water is added on the cotton which is not renewed during this 30 day period; and dead flies are removed every week only, which means that there are almost permanently hundreds of dead flies at the bottom of the cages. When preparing the eggs for the next generation, they are not counted individually, but spread on a graph grid. After the eggs have been spread in a thin layer, and knowing that each square centimeter corresponds to approximately 1200 eggs, it is much faster and easier to count, but less precise. The density of larvae developing in the root could thus be much higher than what we think, if the eggs are not spread thinly enough.

For all these reasons, we hypothesized that the "ideal" conditions of our low-density cage experiment might have given a certain advantage to *Wolbachia*-infected flies, explaining the rapid spread of the bacteria in the experimental cages *vs* laboratory stock.

However, to conclude with certainty that this difference of care could explain the difference we observed, we also conducted PCRs on 83 randomly sampled individuals of the routine laboratory stock at the end of our cage experiment, to check if the infection rate was still at about 50%. To our surprise, it had also increased to 86 % [CI: 76 – 92 %]. We investigated several hypotheses that could have explained this increase in *Wolbachia*-

infected flies over one year: (i) change of temperature or (ii) humidity in the room, (iii) change of supplier/quality for the vermiculite or (iv) swede roots. Unfortunately, we did not find any environmental modification of our rearing conditions that could explain such an increase of the infection rate. However, one explanation could be the "human" factor. At the time we began the cage experiments, a new laboratory technician joined the team and started taking care of the laboratory stock (but note that this technician did not take care of the cage experiment). Although the lab stock protocol stayed the same as before, there are things he might have done differently. For example, as mentioned earlier, eggs that are deposited on the swede roots are not counted individually, but spread on a graph grid for faster counting. When spreading them, he might have been extra careful doing so, managing to do extra thin layers of eggs, which could have reduced the density of larvae within the roots.

Larval density is a potentially important factor because a low density might favor the proliferation of *Wolbachia*. Indeed, theoretical studies show a number of mechanisms by which density-dependent dynamics in the host population can alter the invasion potential of vertically transmitted microorganisms (Altizer & Augustine, 1997; Hancock *et al.*, 2011). Specifically, an applied study demonstrated that the mosquito *Aedes aegypti* population growth was regulated by strong larval density-dependent variation in mosquito fitness components. This density-dependent competition in populations reduced the speed at which *Wolbachia* could invade them at high larval densities (Hancock *et al.*, 2016). Also, we found evidence that *Wolbachia* affects larval fitness components in *Delia radicum*. In Chapter 1, we showed that *Wolbachia* seemed to play a positive role for larvae, and we suggested, following Hosokawa *et al.* (2010) and Nikoh *et al.* (2014), that the bacteria might confer nutritional benefits to the larvae, supplying them with nutrients or vitamins for example, which would enhance larva-to-adult survival in *D. radicum*. When the population density is low (and therefore, a lot of food is available for the larvae), this positive effect of *Wolbachia* might be increased by this lack of competition, sufficiently enough to confer a significant benefit to *D. radicum* infected larvae, which could explain the rapid spread of the infection in both our populations (the experimental ones as the eggs were individually counted, and the laboratory stock ones as the new technician might have been extra thorough with the "thin egg spreading"). However, the opposite could be also true as *Wolbachia* could also intervene to help its

host assimilate nutrients in the case of a low-food supply. An easy way to clarify these hypothesis would be to design a similar experiment following infection rates in different populations, by including in the protocol a "larvae density within the roots" variable.

Our experimental data seemed globally consistent with the simulated data obtained by our model because they also showed an increase of the infection rate (Table 3). However, infection levels were superior to 80% in our three cages after only eight generations, which seems suspiciously high compared to the predicted trend (Figure 4). We therefore went further into assessing the fit between observed and simulated results.

To do so, we fitted a statistical model to our experimental data from the three cages (logistic GLMM with generation as independent variable, cage as a random factor, as well as an individual random factor accounting for residual overdispersion).

This allowed us to compare simulated and experimental invasion dynamics under laboratory conditions (Figure 5). To calculate the goodness-of-fit of experimental data to simulated data, we used the following criterion:

$$\sum_{j=1}^{1000} (pred.simulated - pred.experimental)^2$$

which is the sum of squared errors between infection rates predicted from simulation data and infection rates predicted from experimental data (predictions computed from the same 1000 values regularly spaced between 0 and 8 generations – the total number of generations in the experiment).

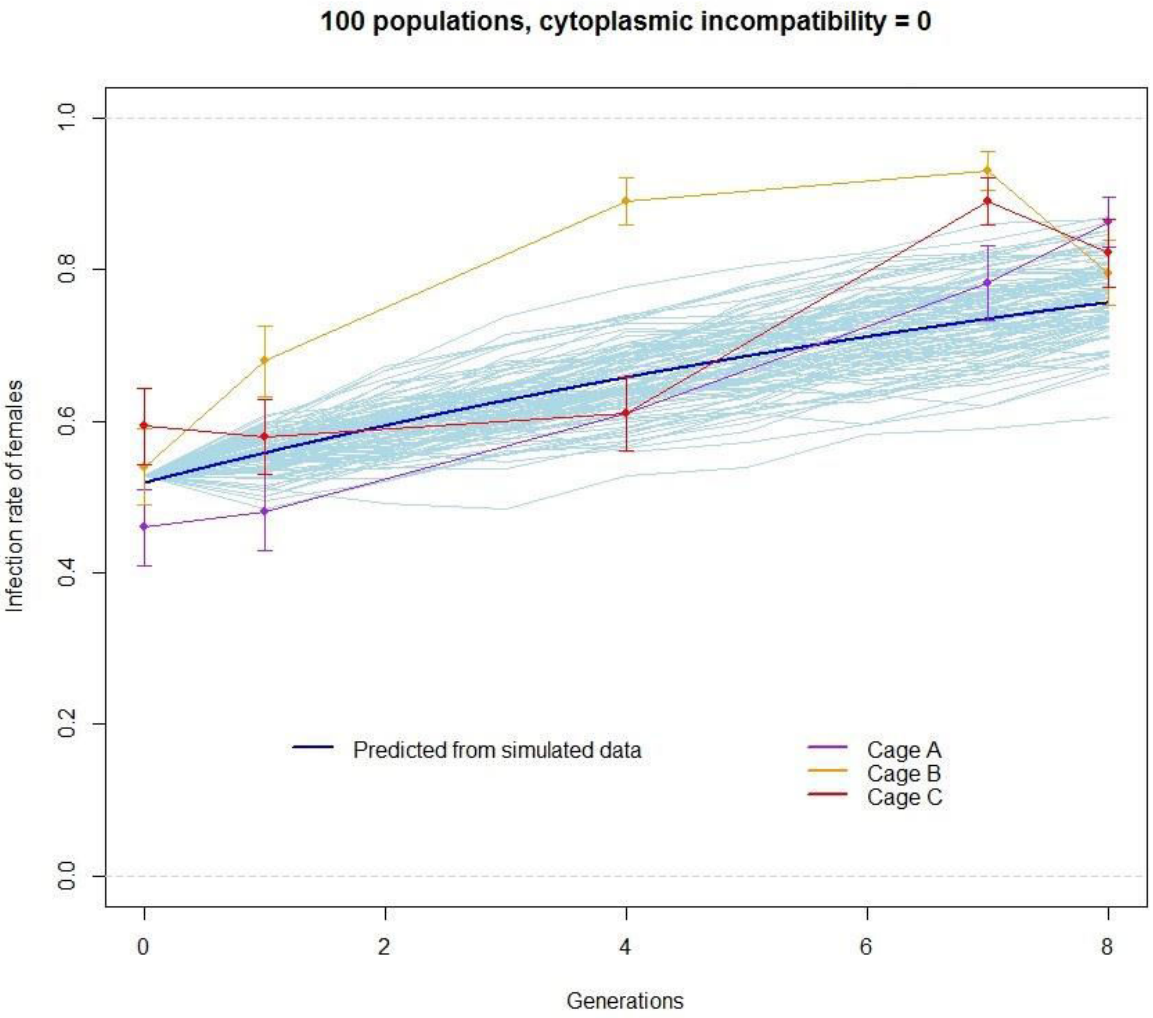


Figure 4. Simulated invasion dynamics of *Wolbachia* in *Delia radicum* after eight generations under laboratory conditions, assuming perfect vertical transmission, no cytoplasmic incompatibility and life history trait estimated values as per Chapter 1. Thin clear blue lines represent the 100 populations simulated, and the dark blue line, the asymptotic model fitted on their averages. Points denote the real infection rates of females in each cage with error bars indicating standard errors.

The simulations based on LHTs data from Chapter 1 predicted a rapid invasion of *Wolbachia* in the population (blue curve), and that is indeed what the experimental data also show (green curve). However, the latter predicts an even faster invasion, which means we might have missed something in our LHTs measurements.

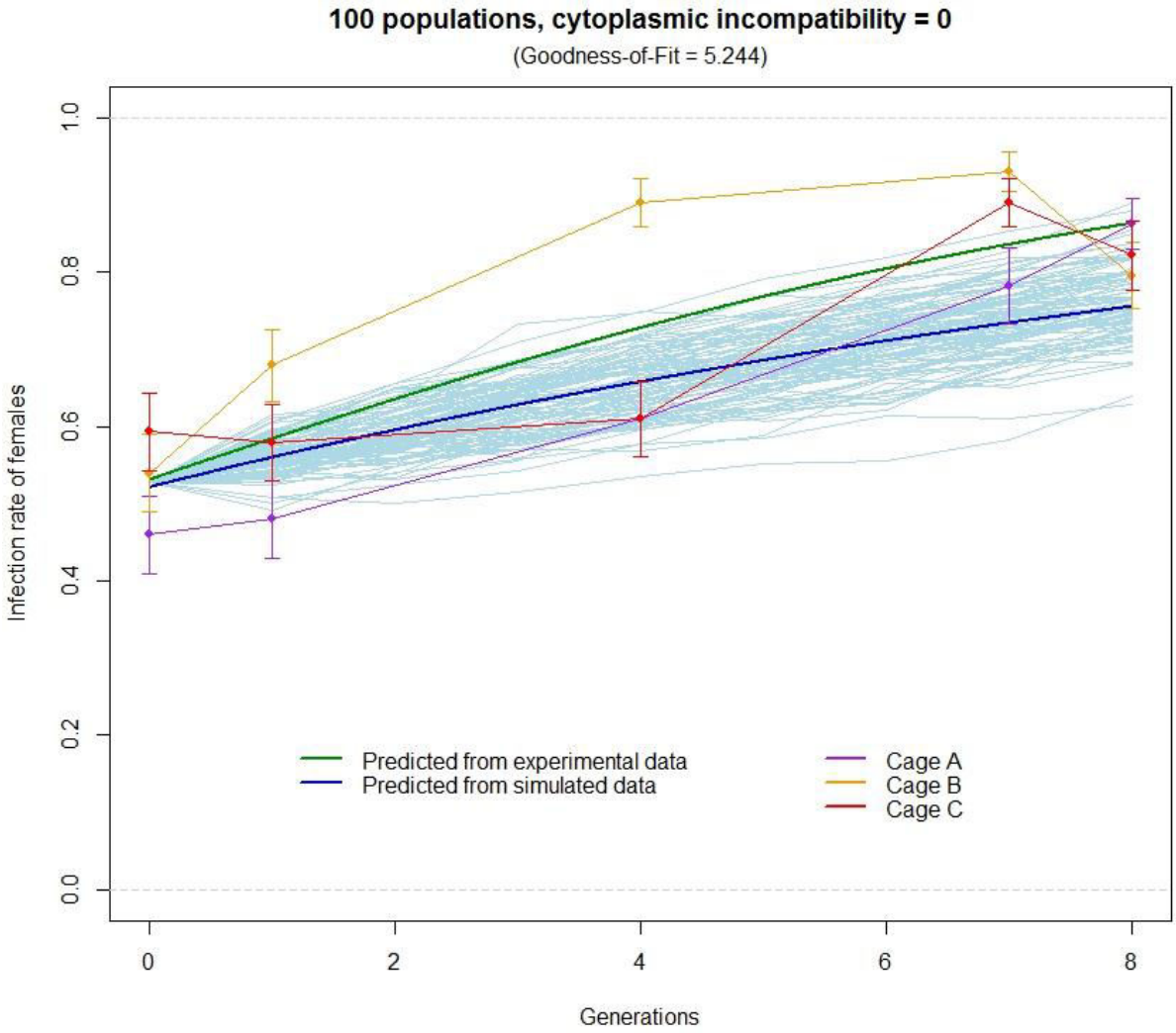


Figure 5. Simulated and experimental invasion dynamics of *Wolbachia* in *Delia radicum* under laboratory conditions, assuming perfect vertical transmission, no cytoplasmic incompatibility and life history trait estimated values as per Chapter 1. Green and blue curves indicate model-based predictions, based on experimental and simulated data, respectively. Points denote the infection rates of females in each cage with error bars indicating standard errors. Thin clear blue lines represent the 100 populations simulated, and the dark blue line, the asymptotic model fitted on their average.

Could a low CI explain the difference between simulated and experimental data?

Because we had assumed an absence of CI in Chapter 1 due to a stable infection rate in our laboratory at the time of the experiment (as well as small and compensating effects of *Wolbachia* on the phenotype of *D. radicum,* which did not make CI necessary to

maintain the infection), we have not experimentally measured it in this population using crosses between infected males and uninfected females. However, the abnormally fast increase of the infection frequency in our cage populations suggested the possibility that a weak but significant cytoplasmic incompatibility could be expressed in this species when the stock was maintained in ideal rearing conditions.

In previous modeling studies, Hoffmann *et al.* (1990) and Turelli & Hoffmann (1995) showed that a stable polymorphic equilibrium, where both infected and uninfected individuals coexist in the same population, is possible if the level of CI just compensates for an incomplete maternal transmission and for any deleterious effects on its female host. In our population, since maternal transmission is complete and since there are no major deleterious effect on *Wolbachia*-infected females, we reasoned that even a low level of cytoplasmic incompatibility would lead to a rapid spread of *Wolbachia*. We therefore calculated what level of CI would have been necessary to explain the gap between our observations and the values predicted from our LHT model alone.

Below is the two-steps process we used to calculate an optimal cytoplasmic incompatibility value:

(i) for each value of CI between 0.07 and 0.20 (realistic CI values according to our different modeling tests), we performed five simulations (with 100 populations simulated per simulation) which each led to a goodness-of-fit value as explained above. The stochasticity included in the model leads to five slightly different values of the goodness-of-fit statistics for each given CI value. This resulted in 70 simulations (14 CI values x 5 replicates per value), then 70 values of goodness-of-fit;

(ii) a quadratic regression was then fitted on the 70 values generated at the previous step and its lowest point taken as the best estimate of CI.

This allowed us to calculate that a CI of about 13-14% would allow our experimental data to fit almost perfectly the simulated model constructed from the LHTs we measured in Chapter 1 (Figures 6, 7).

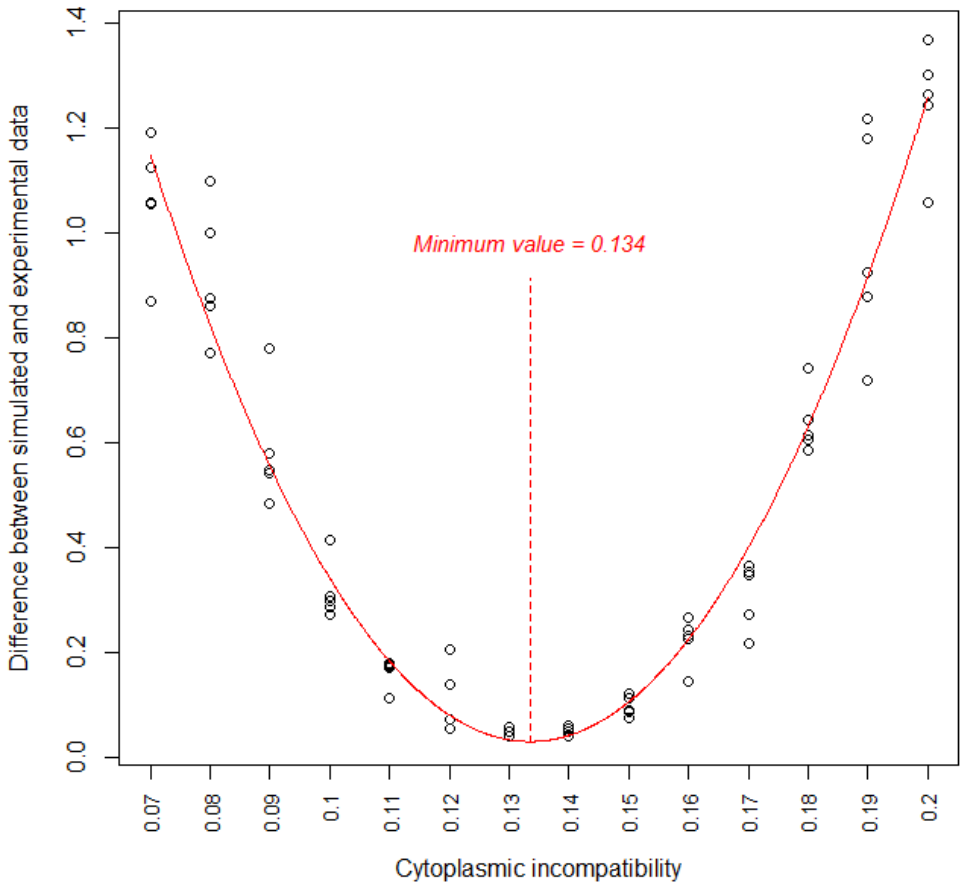


Figure 6. Quadratic regression fitted on goodness-of-fit statistics obtained from 70 simulations varying in their cytoplasmic incompatibility level. The red dotted line shows the minimum value of the curve (0.134), meaning that a CI of about 13-14% would best fit simulation data to our experimental cage data.

Although such a moderate CI would explain the gap between our data and the initial LHT model, this parameter still needs to be experimentally measured by controlled crosses between infected and uninfected individuals to conclude with certainty. Moreover, even if *Wolbachia* does induce some CI in this strain, it does not exclude other traits the bacteria could also have an effect on to increase its prevalence. Overall, what our experimental and simulated results tell us is that:

(i) *Wolbachia* does influence some LHTs in *D. radicum* so that it increases in frequency at least in our experimental cage conditions;

(ii) the 10 LHT traits we measured in chapter 1 are apparently not sufficient to completely explain this increase;

(iii) if CI exists in this strain, then it should be rather low (our best estimate being 13-14% egg mortality – on top of the natural egg mortality – to be consistent with our modelling experiment), and only expressed in very favorable rearing conditions (otherwise our lab strain would have been entirely infected a long time ago).

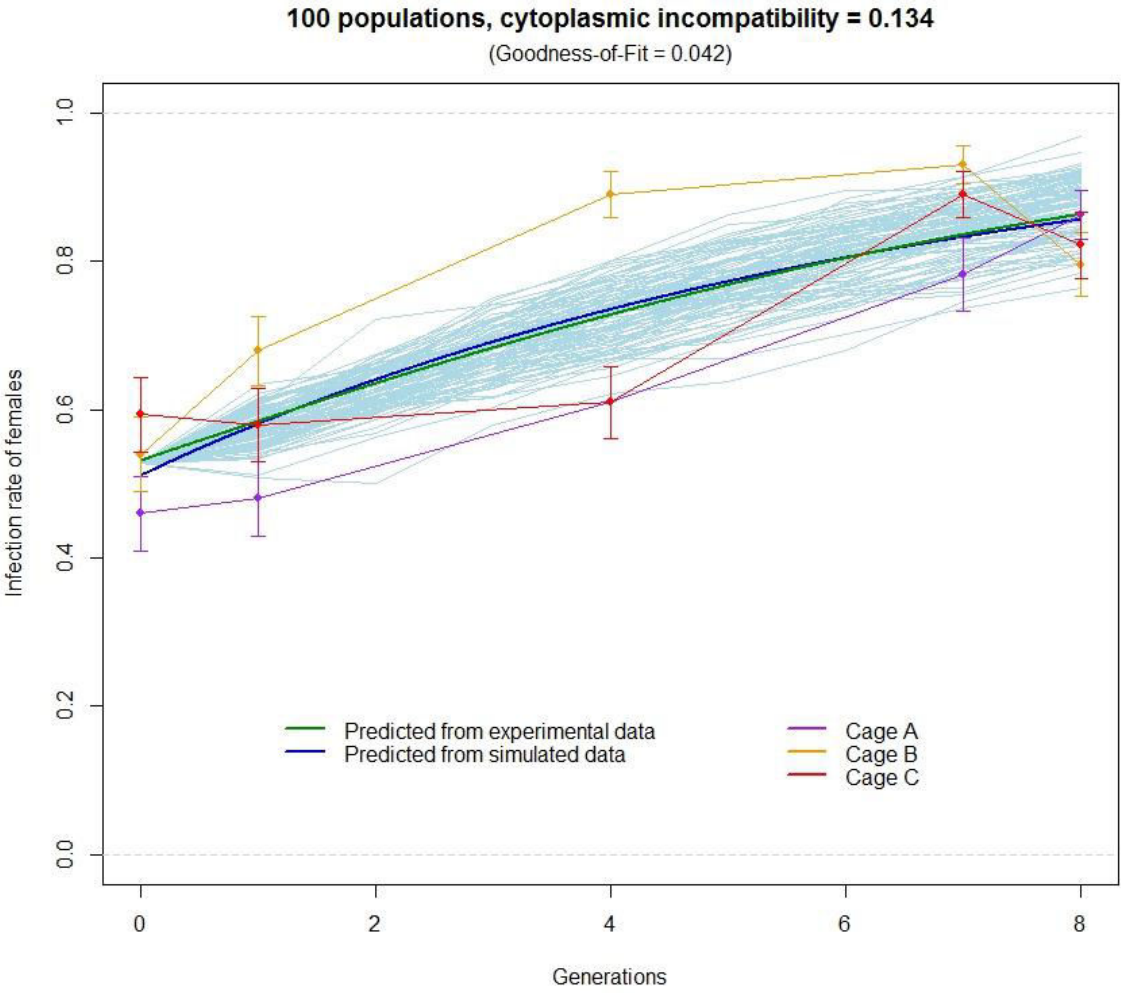


Figure 7. Simulated and experimental invasion dynamics of *Wolbachia* in *Delia radicum* under laboratory conditions, assuming perfect vertical transmission, a 13.4% cytoplasmic incompatibility and life history trait estimated values as per Chapter 1. Green and blue curves indicate model-based predictions, based on experimental and simulated data, respectively. Points denote the infection rates of females in each cage with error bars indicating standard errors. Thin clear blue lines represent the 100 populations simulated, and the dark blue line, the asymptotic model fitted on their average.

However, we must interpret these results with caution. Indeed, this whole study concerns only one population in Brittany. Moreover, our experiments were made in the laboratory only, when we know that conditions in the field can be very different (indeed, our field data suggest a low infection level). This study thus gives us good hints on what *Wolbachia* is able to influence in this species, but it also opens many doors on what could be experimented next.

First, as vertical transmission of *Wolbachia* tends to be better in laboratory stocks than in the field (Hoffmann *et al.*, 1990), it should be checked in nature. A lower transmission could explain partly the low prevalence we observed in the field. However, as poor transmission would quickly lead to the loss of the symbiont, its maintenance in populations – even at a low prevalence – probably requires at least some positive or manipulative effects in the host to compensate losses at each generation (Hoffmann *et al.*, 1990, 1998; Turelli & Hoffmann, 1995; Werren, 1997). Obviously, the next experimental step would be for us to check for the existence of CI in this species using crosses between infected and uninfected individuals.

We also know that the effects of *Wolbachia* can be variable across a range of environments. For example, in *Drosophila melanogaster*, the symbiont increases the fecundity of females during periods of nutritional stress – when subjected to low or high iron environments (Brownlie *et al.*, 2009). It would thus be desirable to check the influence of *Wolbachia* on *D. radicum* by testing other parameters, and more specifically, stressful ones. For example, we could experiment the effects of the symbiont under conditions of abiotic stress such as hot or cold environmental temperatures; but also biotic stress, when the *D. radicum* is exposed to parasitoids. Indeed, this has been observed before in another "host-species couple", as the pea aphid (*Acyrthosiphon pisum*) hosts a symbiont (known as X-type) that can protect it against both parasitoid wasps and heat stress (Heyworth & Ferrari, 2016).

Overall, the lack of any spectacular effect of *Wolbachia* on *D. radicum*, as well as its low prevalence in nature, means that, at this early stage at least, this symbiont does not seem a promising candidate to be used as a lever to control this important agricultural pest.

Gut microbiota perturbation influences life history traits of *Delia radicum*...

In the second part of our thesis, we investigated how an antibiotic treatment targeting the gut microbiota of *D. radicum* would impact LHTs of the fly, using a protocol which allowed to isolate the direct effects of tetracycline (toxic chemical effect) from its indirect effects (due to the partial or total loss of gut microbiota). This time, we used a *Wolbachia*-free strain for the experiments to ensure that the possible effects observed would be due to the modification of the gut microbiota only.

We found that an antibiotic treatment on *D. radicum* adults led to multiple and mostly negative effects on LHTs of their offspring, some of which could happen two generations after treatment. Moreover, even though some negative effects might have been caused by a direct toxicity of tetracycline, most of the differences in the LHTs could only be explained by perturbations of the gut microbiota of the adults treated.

What we observed is actually what we expected when we started the experiment. Indeed, it is now widely known that microorganisms can both synthesize essential nutritional compounds and increase the efficiency of nutrient digestion and absorption by their host (Fraune & Bosch, 2010; Moran, 2007). It has also been shown that gut microbiota could contribute up to 70% of a vertebrate's energy needs (Flint *et al.*, 2008). Therefore, a drastic gut flora change after antibiotic treatment is very likely to damage digestive functions, causing a reduced performance of individuals, and our question was not so much if but which traits would be affected, and by how much.

... with long-lasting effects across generations

Another remarkable result was that this perturbation of gut microbiota seemed large enough to be detected in the offspring of the individuals treated and, in some case, their grandchildren. Because we only used tetracycline in this study, and because we know that some bacteria strains are resistant to this antibiotic (Roberts, 1996), we suggest that some tetracycline-insensitive bacteria taxa probably survived the treatment during the experiment, and multiplied to occupy the niches liberated by the antibiotic treatment. Therefore, offspring and grandchildren probably had a microbiota very different from those of their ascendants. Considering that the inclusive fitness of an individual

(Hamilton, 1964) takes into account not only its offspring number but also their own fitness, the maternal and grandmaternal effect of a microbiote perturbation underlines the importance of that vital component of the holobiont concept (Mindell, 1992).

How is the microbiota of the offspring established and to what extent is it perturbed when ascendants are treated?

Unfortunately, one of the main limit of this study is that the discussion of our results remain hypothetical because we do not know how much the gut microbiota was modified by the treatment and how much of it was transmitted across the generations. To complete these results, we would need to know the richness and diversity of gut microbiota in our four kinds of treatments. However, this was part of our initial plan; accordingly, during the experiment, we kept dead adults individually in 96° alcohol at -20°C, to preserve their DNA. After extracting it, we ran PCRs on each of the flies by using 16S primers to check for bacterial DNA presence, and sent amplicons to sequencing. At the moment of writing, the results are still being analyzed. In any case, they should be very informative on what happened to the gut during this experiment. Also, even though we only have LHTs measurements for the last generation (offspring), we kept and sequenced the two previous generations as well (parents and grandparents) which should allow us to trace very nicely the evolution of gut microbiota through the different treatments and generations.

Which bacterial taxa influence the extended phenotype of the host?

Ideally, we would like to link some bacterial taxa to their possible contribution to a specific host phenotype. However, this might be more difficult than planned as we know that the gut microbiota can vary not only in total size but also in composition between species or populations (Colman *et al.*, 2012; Staubach *et al.*, 2013). For example, Wong *et al.* (2013) explored the gut microbiota of 11 species of *Drosophila* but did not find any evidence of a core "drosophila" gut microbiota between them. On the contrary, Broderick and Lemaitre (2012) reviewed different studies that investigated the composition of gut

microbiota in *Drosophila* and found that both for laboratory stocks and wild-caught flies *Lactobacillus plantarum* and *Acetobacter pomorum* were always present. However, since diet is one of the main factors driving variation in the composition of the gut microbiota (Lozupone *et al.*, 2012), regardless of taxonomy and geography (Chandler *et al.*, 2011), we think that we might still be able to find similarities between our individuals' microbiota, and that some patterns might be revealed anyway. Indeed, all flies have been reared in the exact same conditions – except for the tetracycline treatment – with the exact same food.

Thanks to new molecular data mining techniques such as PICRUSt, it is now possible to predict and compare probable functions across many DNA samples using the phylogenetic information contained in 16S marker gene sequences (Langille *et al.*, 2013). The PICRUSt package automatically scans available databases to predict metagenome functional content from marker gene (e.g. 16S rRNA) surveys and full genomes. Thanks to this technique, it is thus possible to predict functional potentials of the bacterial community associated with different individuals (Figure 8). For example, it can identify bacterial communities rich in genes involved in membrane transport, amino acid metabolism, carbohydrate metabolism, or even replication and repair processes (Kaczmarczyk *et al.*, 2018). This new technique would be perfect to analyze our results as we would like to investigate which strain of bacteria is associated with a certain type of phenotype or function.

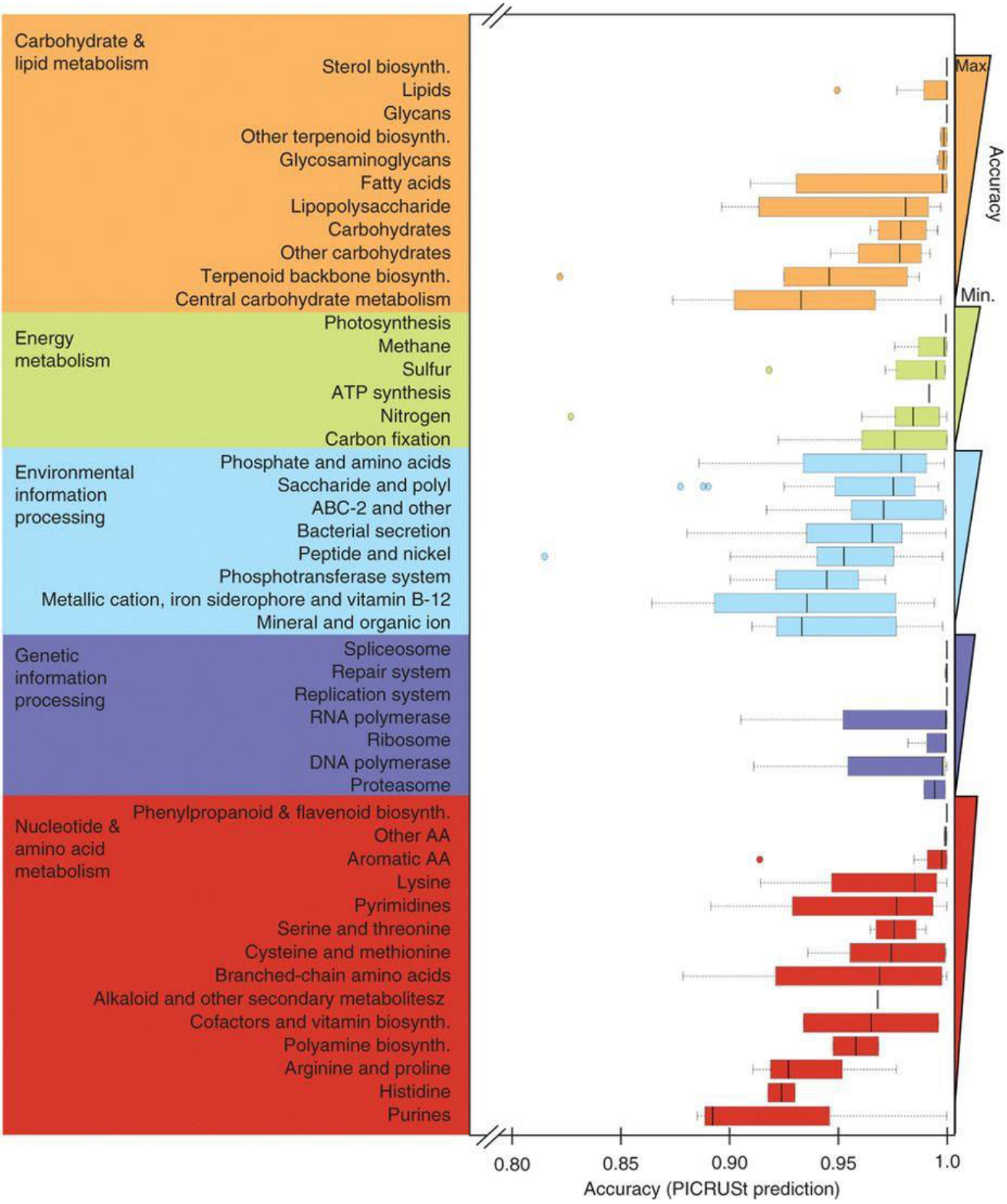


Figure 8. Typical output obtained with the PICRUSt software: predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Results are colored by functional category and sorted in decreasing order of accuracy within each category (indicated by triangular bars, right margin). Note that accuracy is >0.80 for all, and therefore the region 0.80–1.0 is displayed for clearer visualization of differences between modules (from Langille *et al.*, 2013).

How does *Delia radicum* acquire its microbiota?

The only study that extensively described *D. radicum* microbiota, thanks to sequencing of whole individuals, found that its bacterial diversity was comparable to other studies on insects, with around 16 identified genera (Bili *et al.*, 2016). However, the authors stipulate that some rare bacteria present at a low density in their samples may have been missed due to the high density of some bacteria taxa dominating each sample. In *D. radicum*, the bacterial communities were quite similar within populations from different geographic areas (although they were still all sampled in Brittany). Another study made in Canada, using a culturing approach on midgut and fecal materials, identified nine types of culturable bacteria (Lukwinski *et al.*, 2006). However, none of theses studies allows to establish how the microbiota of this species is established in the first place (i.e. which part is inherited and which part is acquired from the environment).

Therefore, we have launched another study (still ongoing at the moment) to enrich our knowledge on this subject. Because our tetracycline experiment had led us to suspect that at least a significant part of the gut microbiota of *D. radicum* is maternally inherited, we designed a protocol to characterize and study the heritability of the fly microbiota, as well as its establishment through the different developmental stages of *D. radicum*. The aim was to determine which part (if any) of the gut microbiota is maternally inherited and which part is acquired from the environment when *D. radicum* larvae feed in the rhizosphere.

For this experiment, we used a *Wolbachia*-free *Delia radicum* population to be relevant with our previous study on tetracycline. To isolate the potentially heritable fraction of the microbiota, eggs were collected after females laid on swedes root which surface had been sterilized using sodium hypochlorite. Some of these eggs were immediately stored for future analysis and the others were deposited on the stems of 7-week-old oilseed rape plants (*Brassica napus L. subsp. oleifera*). We then sampled the plant leaf, plant collar, plant root, rhizosphere, bulk soil, and insect along each stage of *D. radicum* development (Figure 9).

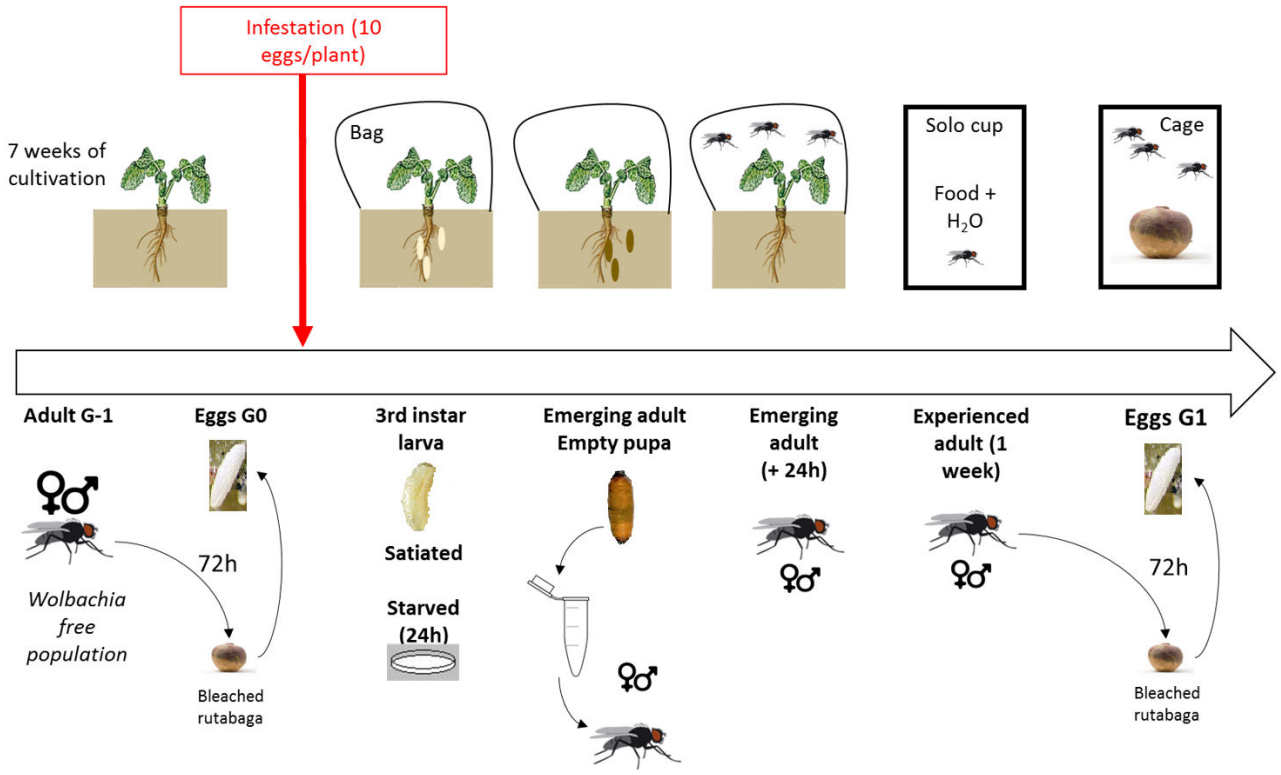


Figure 9. Schematic representation of the protocol (modified from Morgane Ourry's illustration).

This study should allow to answer several questions: (i) which part of the larval and of the adult microbiote is maternally inherited (via the envelope of the egg)? (ii) is the non-inherited part mostly typical from the plant or from the soil community? (iii) to what extent are the larval and the adult gut microbiota taxonomically and functionally similar? (iv) which developmental stage hosts the more diverse microbial communities?

At the moment of the writing of this manuscript, all the plant, soil and insect samples have been collected and their DNA have been extracted for sequencing, which is ongoing.

Endocellular symbionts as participants in the insect-plant dialogue

In the third and last part of our thesis, we investigated whether *Wolbachia,* despite being endocellular, could have an influence on a third trophic level, which is the plant its host feeds on. Indeed, some studies showed recently that insect microorganisms are actually important "hidden players" in insect-plant interactions, and can manipulate plants, generally to the benefit of their insect host (Kaiser *et al.*, 2010; Frago *et al.*, 2012; Body *et*

al., 2013; Giron *et al.*, 2013, 2016, 2017; Sugio *et al.*, 2015). This can also be true for endocellular symbionts (Su *et al.*, 2015) including *Wolbachia*, which has been shown previously to lower plant defenses attacked by its host (the beetle *Diabrotica virgifera virgifera*) (Barr *et al.*, 2010), or to maintain "green islands" (small patches than remain photosynthetically active on senescent leaves) which are beneficial to its leaf-mining moth host *Phyllonorycter blancardella* (Giron *et al.*, 2007; Kaiser *et al*., 2010).

By infesting oilseed rape (*Brassica napus L. subsp. oleifera)* plants with *Wolbachia* -infected and -uninfected larvae of *D. radicum*, we compared direct (glucosinolates levels) and indirect (volatiles) plant defenses between both treatments. Our main result was that the leaf glucosinolate content decreased significantly in plants attacked by *Wolbachia*-infected larvae, particularly for three aliphatic compounds. This shows that *Wolbachia* has indeed an influence on the defense reaction of *D. radicum* host plant. Better, this type of modification is probably advantageous for *D. radicum* because a decrease in aliphatic glucosinolates in the leaves probably translates into a decrease of associated isothiocyanates (Vig *et al.*, 2009), volatiles which can attract the fly natural enemies (Neveu *et al.*, 2002; Ferry *et al.*, 2007; Soler *et al.*, 2007) but also conspecific and heterospecific competitors (Finch, 1978).

Even though the limited number of replicates of our experiment may not have supplied sufficient power to detect moderate differences (especially for the volatiles experiment), the effects we did find confirm that the field of insect-plant interactions must now take intracellular microorganisms into account in future studies because of the complex interactions taking place between the three trophic levels plant-insect-symbiont.

Now that we know that *Wolbachia* can influence the plant defenses in response to a *D. radicum* host attack, the next step would be to understand the mechanisms. In Chapter 3, we suggested the following two hypotheses.

An indirect effect by modifying the gut microbiota?

Wolbachia could influence the reaction of plants indirectly through a modification of the gut microbiota of its host. This hypothesis assumes that microbiota products found in the feces of *D. radicum* larvae provide a cue to the plant and prime its defense. If *Wolbachia*

was able to alter this signal, it may modify the reaction of the plant to the advantage of the host. Such an impact of *Wolbachi*a on a gut microbiota composition is not a purely theoretical possibility since it has been demonstrated recently in *Drosophila melanogaster* (Simhadri *et al.*, 2017; Ye *et al.*, 2017).

Modifying the gut microbiota of the host by *Wolbachia* could however also go in the wrong direction since this bacterial community can directly help the host to neutralize plant defenses. Because toxic isothiocyanates are liberated upon feeding of *D. radicum* larvae (Crespo *et al.*, 2012; van Dam *et al*., 2012) a defense mechanism against isothiocyanates is necessary. This mechanism was partly unraveled in a recent study which showed that the cabbage root fly larval gut microbiota was capable of isothiocyanate degradation thanks to the microbial enzyme SaxA, found in several gut microbial taxa (Welte *et al.*, 2016). The gut microbiota of *D. radicum* thus seems to play a role in the detoxification of the plant toxins, and might make it essential for *D. radicum* survival. Any modification of the larval gut microbiota by *Wolbachia* would therefore be selected against if it impacted this key function. On the other hand, any *Wolbachia*-related modification of the larval gut microbiota which would favor Sax-A-capable taxa would be selected for. A direct demonstration of *Wolbachia* altering the microbiota of *D. radicum* could be achieved by comparative 16S rDNA gene sequencing of microbiota in *Wolbachia*-infected and *Wolbachia*-free individuals. One of the goals of this comparison would be specifically to check if taxa producing Sax-A are still present in individuals carrying *Wolbachia*. This analysis is currently ongoing and our results should be available soon.

A direct effect through a modification of the host saliva?

Another non-exclusive hypothesis would be that *Wolbachia* directly influences the plant response via elicitors discharged in the saliva of its host. It was shown for example that when feeding, the Colorado potato beetle *Leptinotarsa decemlineata* releases bacteria in its oral secretions which disrupt phytohormone expression and suppress induced plant defenses (Chung *et al.*, 2013). As *Wolbachia* have repeatedly been found in the cells of insect saliva glands (Wolstenholme, 1965; Zouache *et al.*, 2009; Amuzu *et al.*, 2015), it is possible that the symbiont could modify saliva composition *in situ*, which could in turn modify plant response, as it has been shown already (Chung *et al.*, 2013). Another

perspective could therefore be to screen *Wolbachia* in insect tissues of *D. radicum*, and to search specifically for its presence in salivary glands. Through the use of two techniques based on staining and microscopy, is now possible to obtain very precise images of the location of *Wolbachia* (or other bacteria) in insect tissues.

A first method is Fluorescence In Situ Hybridization (FISH), and is based on the fluorescein-labeled oligonucleotides developed by Xi *et al.* (2005). It was developed to detect bacteria inhabiting microarthropods by means of small-subunit rRNA-targeted fluorescence *in situ* hybridization and microscopy. This protocol is based on cryosections of whole specimens (Thimm & Tebbe, 2003) (see example in Figure 10).

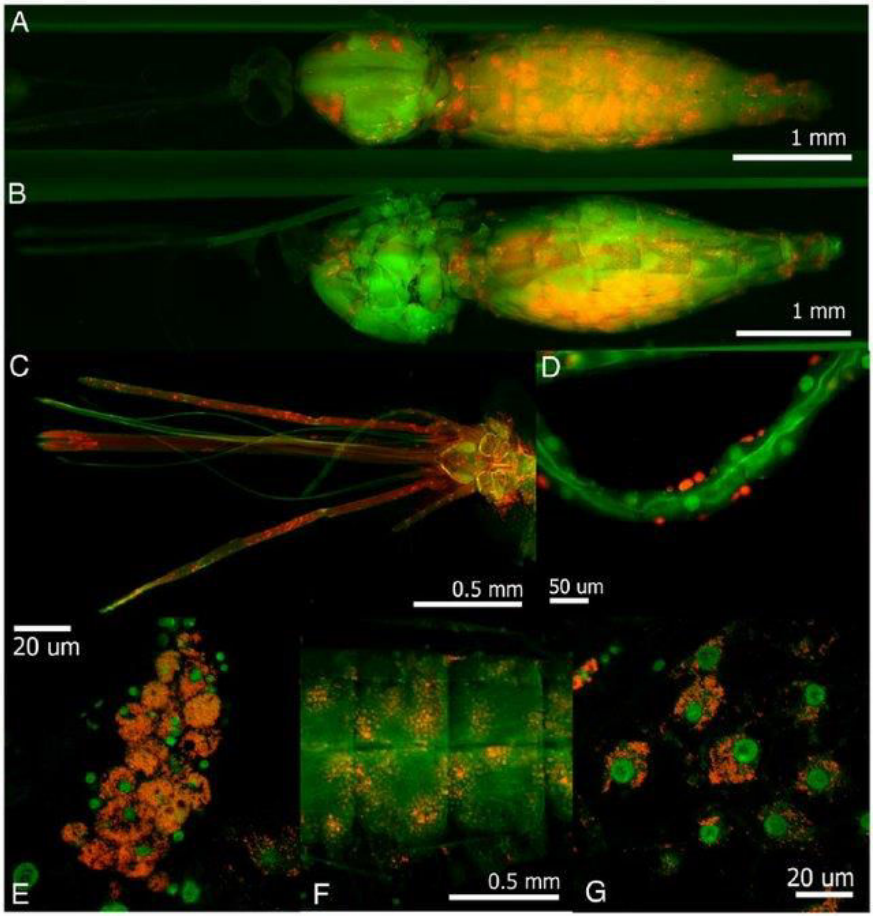


Figure 10. Example of FISH staining (from Hughes *et al.*, 2011). Whole mount fluorescence in situ hybridization of *Wolbachia*-infected *Anopheles gambiae*, 30 days post-injection. *Wolbachia* is distributed throughout the mosquito. Red: *Wolbachia*. Green: mosquito cell nuclei. (A) Dorsal view of whole mosquito. (B) Lateral view of whole mosquito. (C) Head, mouthparts and antennae of the mosquito. (D) Hemocytes adhering to Malpighian tubules. (E) Fat body (F) Abdomen from ventral view. (G) Intracellular *Wolbachia* infecting cells.

A second method uses Syto-11, a cell-permeant fluorophore with high affinity for double-stranded DNA. Syto-11 can be used to stain free-living bacteria, and even intracellular ones such as *Wolbachia*. This method was notably used to stain *Wolbachia* in *Drosophila* eggs (Albertson *et al.*, 2009) (Figure 11). The advantage of this staining approach is that it informs about *Wolbachia* titer as well as its tissue and cellular distribution (Casper-Lindley *et al.,* 2011). In a recent study, both methods were compared to stain *Wolbachia pipientis* in cell cultures (Venard *et al.*, 2012), and the authors suggested to preferably use Syto-11 to study *Wolbachia*'s biology in live studies, since this procedure does not require fixing of the cells.

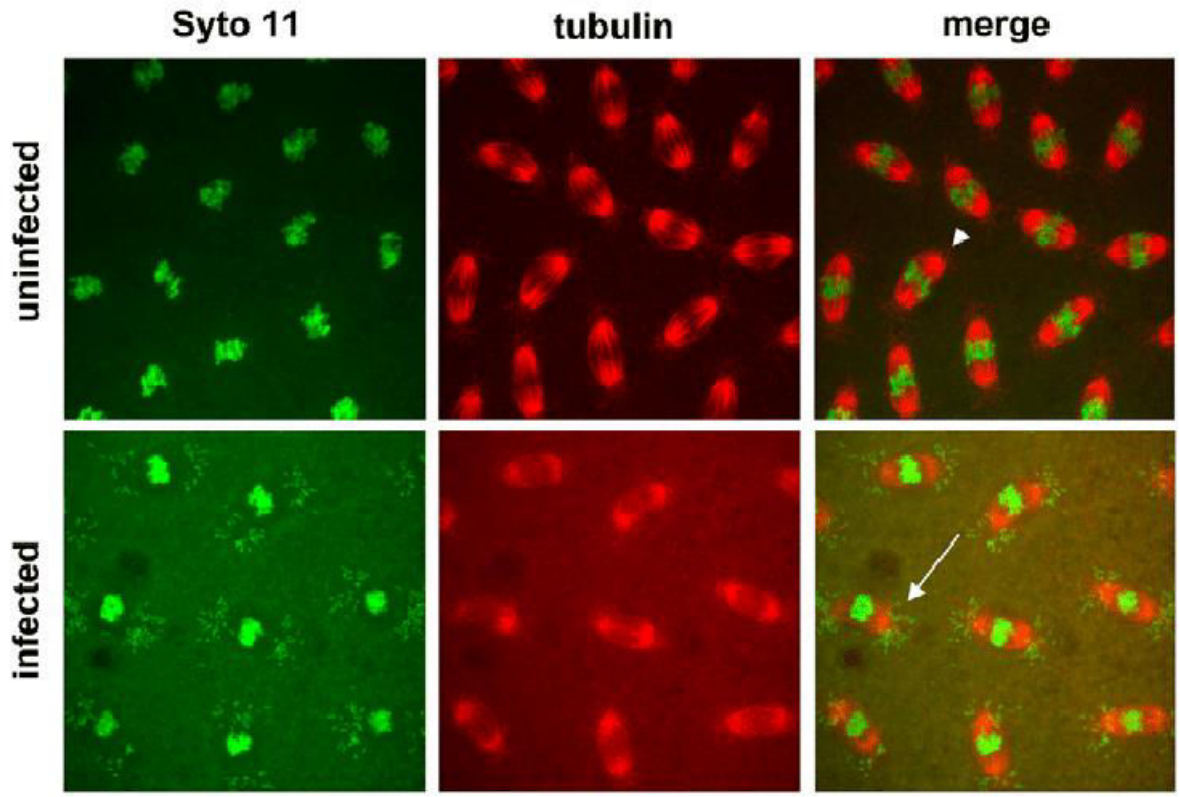


Figure 11. Example of Syto-11 staining (from Albertson *et al.*, 2009). Syto 11 labels *Wolbachia* and nuclear DNA in *Drosophila* embryos. Fixed uninfected and infected *D. simulans* embryos stained with Syto 11 (green) to label nucleic acid and α-tubulin (red) to label microtubules. *Wolbachia* are present as rod-shaped particles (arrow) located at the poles of the mitotic spindle in infected embryos and are absent from uninfected embryos (arrowhead).

Conclusion

In this thesis, we have aimed at studying the holobiont constituted by *Delia radicum* and its symbionts. This work is original in the sense that we tried to discuss here not only host-symbiont relationships but also the tri-trophic interactions between oilseed rape, the cabbage root fly and its symbionts (Figure 12). We have shown that bacteria, whether they live directly within the cells or in the gut, can have various effects on their host, from reducing survival in stress condition to enhancing developing larva viability. Their influence is even greater in the sense that when the microbiota is perturbed, the consequences can be seen in further generations. Moreover, symbionts not only influence their insect hosts, but are also able to manipulate other trophic levels, such as the plants.

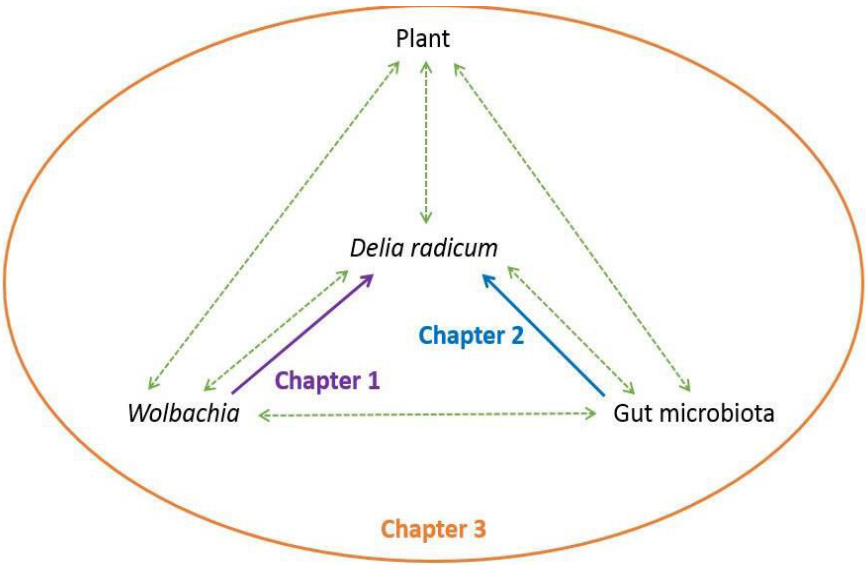


Figure 12. Schematic representations of all the possible interactions between the species considered in this thesis (dashed green arrows). Purple, blue and orange arrows show the specific interactions studied in this thesis.

The main picture that emerges from this work is that whatever their location (gut lumen or host cell), bacterial symbionts are able to interact with many physiological and ecological processes, on more than one trophic level (not to speak about interactions between the cellular and gut compartments themselves). Most studies understandably focus on the effects of one particular category of symbiont on its host, fewer search for the interactions that may take place within an insect between these different types of microorganisms, and fewer still take the plant partner into account. Knowing that the majority of insects feed on plants (Strong *et al.*, 1984), and that all insects have symbionts,

the possible interactions seem unlimited, even more so when we consider how many other "actors" could actually also play a part. For example, we focused in this thesis on the intracellular and gut symbionts, but all the bacteria present in the environment that insects are in contact with could complicate these interactions even more (Figure 13).

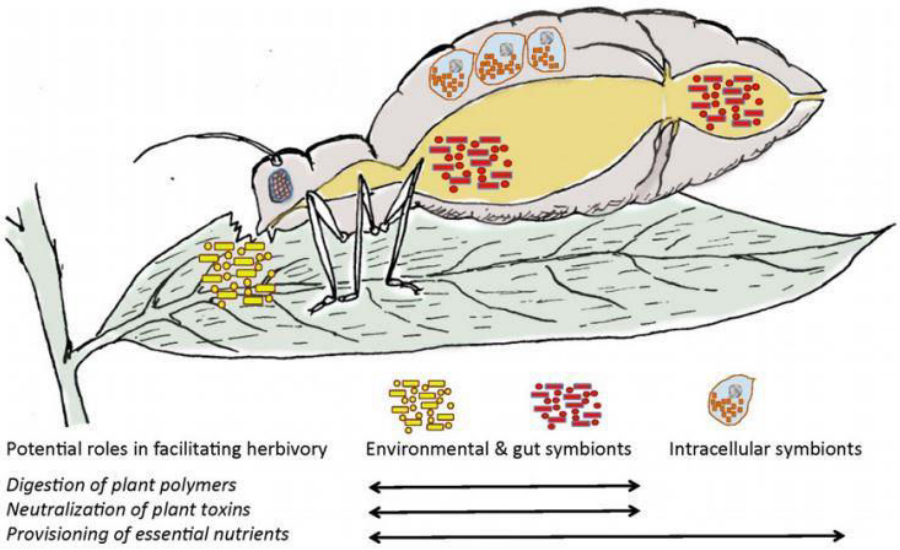


Figure 13. Potential contributions of microorganisms to host ability to feed on live plants depend on the symbiont's location inside or outside the insect body (from Hansen & Moran, 2014).

Taking into account all these compartments in further studies will obviously complicate them. However, this thesis demonstrates that this is now a necessity to unravel possible relationships between different partners, as well as their ecological or evolutionary implications. Moreover, thanks to the molecular tools available now, understanding the cellular and molecular dialogue taking place within the holobiont is within our reach.

Nonetheless, how to interpret the results we might obtain is still under debate. When Lynn Margulis (1993) came up with the term "holobiont" to define a host and its symbiotic microbiota, the term was widely accepted, just as the word "hologenome" to define the union of all the genes in the holobiont, i.e., all the genes in the symbionts plus the genes in the host. Recently, Zilber-Rosenberg and Rosenberg (2008) went further by suggesting the "hologenome theory of evolution": the holobiont with its hologenome, acting in consortium, should be considered as a unit of selection in evolution, and this unit has properties similar to an individual organism, i.e., it is a superorganism. Thus, variation in the hologenome can lead to variation in phenotypes upon which natural

selection can operate. Although this concept redefines what constitutes an individual, it does not change the rules of evolutionary biology. Simply put, if the microbiota is a major, if not dominant, component of the DNA of a holobiont, then microbiota variation can quite naturally lead to new adaptations and speciation, just like variation in nuclear genes (Bordenstein & Theis, 2018).

However, the hologenome theory of evolution has been questioned from the start. Other authors argue that considering the host and its microbiote as an ecological community (and not as a super-organism) is a better approach because a more complete understanding will be gained by considering its members as evolutionary entities that experience different selection pressures, potentially live in a different range of habitats, and vary in their fidelity to the association (Douglas & Werren, 2016). Granted, in the hologenome theory of evolution, partner fidelity (stable association of host and symbiont genotypes across multiple host generations) is a prerequisite (Zilber-Rosenberg and Rosenberg, 2008). However, even when coinheritance occurs for a subset of the microbial associates[1], it is unlikely to be so for all members of the community, and so it seems difficult to imagine that the entire microbiome should be considered part of a "hologenome" with its host if only a subset of microbes meets the requisite conditions (Douglas & Werren, 2016).

Another requirement in the hologenome concept is the perfect concordance of selective interests both among the microbial partners and between the microbiota and host (Zilber-Rosenberg and Rosenberg, 2008). However, it is difficult to imagine that the entire microbiota within a host will have a shared interest as there are often divergent selective pressures of different microorganisms within a single host. For example, antagonistic interactions can occur among different mutualists within the same host (Afkhami *et al.*, 2014; Coyte *et al.*, 2015). There can be thus conflicts at two levels: between the host and its microbial symbionts and among different symbionts (Douglas & Werren, 2016). But does it really mean that a superorganism cannot evolve as a whole nevertheless? Conflicts of interests are everywhere, and even within our own cells when

[1] If we dared to play the word-coining game, we may propose to name "inheritome" or "transmitome" that specific part of the hologenome that is transmitted to the offspring with the same certitude as mitochondria. It would, however, constitute yet another category in what is in reality a continuum between nuclear genes and transient secondary symbionts.

we think about cytonuclear conflicts (since nuclear and cytoplasmic genes usually have different modes of transmission, intragenomic conflicts between them may arise – Murlas Cosmides & Tooby, 1981); still, we evolved.

Therefore, can the hologenome be considered as a unit of selection in further studies? Or is it better to apply to host-microbiota systems "classical" community ecology principles, such as successional theory, multitrophic interactions, and disturbance ecology (Christian *et al.*, 2015)? The question remains open and might gain a lot of interest during years to come.

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Appendix: Methodology for the modeling of a *Wolbachia* infection

1. Modeling of simulated data from 100 virtual populations

To model the *Wolbachia* infection rate of females depending on *Delia radicum* life history traits (LHT), we adapted a model from Farkas *et al.* (2017)[3] and simulated the evolution of the *Wolbachia*-infection rate in 100 independent virtual cage populations, assuming a perfect maternal vertical transmission, no cytoplasmic incompatibility, and the LHT values we had estimated in Chapter 1 (Table 1). To the original, deterministic model of Farkas *et al.* (2017), we added a stochastic component to simulate what happens in finite populations of laboratory-realistic size. This stochastic step consisted in a random sampling of 600 eggs to create Generation$_{T+1}$ among the total number of eggs produced at Generation$_T$.

Table 1. Parameters included in the model to simulate the evolution of *Wolbachia* in our laboratory strain. For each value, u: uninfected and i: infected.

Parameters included in the model	**Values of the parameters**
Initial infection rate	0.53[4]
Numbers of individuals in the initial population	180 males, 180 females[5]
Number of infected males / females	M_i (or F_i) = 180 * 0.53
Number of uninfected males / females	M_u (or F_u) = 180 - M_i
Oviposition probability	γ_u = 0.80 γ_i = 0.83
Fecundity (3 days) in number of eggs	λ_u = 29 λ_i = 27
Sex-ratio (proportion of females)	φ_u = 0.49 φ_i = 0.49
Emergence rate	μ_u = 0.39 μ_i = 0.46
Maternal transmission rate of *Wolbachia*	τ = 1.00
Probability of Cytoplasmic Incompatibility (CI)	α = 0

[3] Farkas, J.Z., Gourley, S.A., Liu, R., Yakubu, A.A. (2017). Modelling *Wolbachia* infection in a sex-structued mosquito population carrying West Nile virus. Journal of Mathematical Biology 75: 621–647.

[4] This infection rate was the last we had measured in our laboratory stock, approximately one month before the start of the modeling experiment

[5] This numbers correspond to a protocol for a real cage experiment we had in mind at the time, and which is presented further below

1.1. Step 1

From the number of (un)infected males and females of the previous generation, we compute the number of eggs produced (for each sex and infection status).

The equations of the model are as follow:

$$M'_u = \frac{M_u F_u}{N}\gamma_u\lambda_u(1-\phi_u) + \frac{M_u F_i + M_i F_i}{N}(1-\tau)\gamma_i\lambda_i(1-\phi_i) + \frac{M_i F_u}{N}(1-\alpha)\gamma_u\lambda_u(1-\phi_u)$$
$$M'_i = \frac{M_u F_i + M_i F_i}{N}\tau\gamma_i\lambda_i(1-\phi_i)$$
$$F'_u = \frac{M_u F_u}{N}\gamma_u\lambda_u\phi_u + \frac{M_u F_i + M_i F_i}{N}(1-\tau)\gamma_i\lambda_i\phi_u + \frac{M_i F_u}{N}(1-\alpha)\gamma_u\lambda_u\phi_u$$
$$F'_i = \frac{M_u F_i + M_i F_i}{N}\tau\gamma_i\lambda_i\phi_i$$

with M'_u: number of uninfected male eggs in the next generation; M'_i: number of infected male eggs in the next generation; F'_u: number of uninfected female eggs in the next generation; F'_i: number of infected female eggs in the next generation; N: total number of individuals (males + females).

Each term of each sum is rounded to the nearest integer value.

1.2. Step 2

Among the total number of eggs produced at step 1 ($M'_u + M'_i + F'_u + F'_i$), we randomly sample 600 eggs that will constitute the basis of the next generation. The number of eggs sampled from each class is noted $M''_u + M''_i + F''_u + F''_i$.

1.3. Step 3

From the 600 eggs sampled at step 2, we compute the number of adults produced:

$$M'''_u = M''_u \times \mu_u$$
$$M'''_i = M''_i \times \mu_i$$
$$F'''_u = F''_u \times \mu_u$$
$$F'''_i = F''_i \times \mu_i$$

Each number is rounded to the nearest integer value

2. Extraction of the general trend from simulated populations

From the 100 simulations obtained previously from our virtual populations (thin blue curves in Figure 3, 4, 5, 7 in the General Discussion), we compute the mean infection of females for each generation; we thus obtain one value per generation.

Then, we fit an asymptotic model on these mean values and we constrain it so that the asymptotic infection rate is comprised between 0 and 1 (dark blue curve in Figure 4, 5, 7 in the General Discussion).

French Summary / Résumé en français

Introduction générale

La symbiose est une association intime et durable entre deux organismes appartenant à des espèces différentes, dont l'un, appelé hôte, héberge l'autre, appelé symbiote. En 1993, Lynn Margulis a proposé que l'assemblage résultant d'un hôte et l'ensemble de ses symbiotes soit appelé "holobionte".

Les associations symbiotiques entre microorganismes et eucaryotes sont omniprésentes dans le monde vivant, animal et végétal. Plus particulièrement, tous les insectes sont colonisés par des symbiotes, pouvant être situés dans le microbiote intestinal ou au sein même des cellules des tissus. Ces symbiotes peuvent être de nature très diverse ; le plus souvent des bactéries ou des champignons, mais aussi parfois des parasites ou des virus.

Ces microorganismes peuvent jouer un rôle crucial dans l'évolution et l'écologie de leurs porteurs en modifiant le phénotype de ces derniers. En effet, certains symbiotes intracellulaires peuvent améliorer l'apport nutritionnel de leurs hôtes, conférer une protection contre les ennemis naturels (virus, champignons ou prédateurs), ou encore modifier certains traits d'histoire de vie, comme la longévité, la taille ou la couleur de leurs hôtes. De même, le microbiote intestinal peut contribuer à la nutrition des hôtes, à leur protection contre les parasites et les pathogènes, à la modulation de certaines réponses immunitaires ou parfois même à la communication intraspécifique.

Certains de ces symbiotes étant héritables via la mère, les phénotypes étendus résultant de ces associations symbiotiques peuvent se transmettre aux générations suivantes. L'acquisition d'un symbiote dans une population naturelle peut donc s'apparenter à l'apparition d'une série de mutations génétiques : il s'agit d'un supplément d'information, d'une nouveauté génétique importante. Le symbiote peut en effet apporter une ou plusieurs fonctions « prêtes à l'emploi » à son hôte, pouvant être bénéfique(s) ou pas.

La mouche du chou (*Delia radicum*) est un ravageur majeur des cultures en milieu tempéré. Son microbiote a récemment été décrit, révélant la présence de *Wolbachia*, une bactérie intracellulaire à transmission verticale connue pour manipuler la reproduction de ses insectes-hôtes, mais également pour influencer certains de leurs traits d'histoire

de vie. Or, nous ne connaissons pas encore les effets de ce symbiote chez la mouche du chou. De même, étant donné que les microorganismes (extracellulaires) de l'intestin peuvent aussi influencer grandement certains traits d'histoire de vie de leurs hôtes, une partie de cette thèse est consacrée à l'étude de l'impact du microbiote intestinal sur certains aspects de la biologie et de l'écologie de la mouche du chou (y compris sa relation avec la plante hôte).

Les interactions entre les ravageurs et leurs ennemis naturels étant amenée à devenir un outil de plus en plus important en agronomie (de par la limitation de la lutte chimique), comprendre le rôle exact du microbiote (intestinal et intracellulaire) chez ces espèces est d'une importance cruciale.

Cette thèse présente l'originalité d'étudier pour la première fois simultanément deux compartiments bactériens (intracellulaire et intestinal), ainsi que leurs interactions. Elle s'organise en trois chapitres : (i) dans le chapitre 1, nous décrivons les effets de *Wolbachia* sur *D. radicum*, en analysant certains traits d'histoire de vie «classiques» de la mouche du chou; (ii) dans le chapitre 2, nous appliquons un traitement antibiotique à une souche de *D. radicum* exempte de *Wolbachia*. Nous utilisons un protocole nous permettant d'opposer les effets directs de l'antibiotique sur le phénotype de la mouche (liés à la toxicité de la tétracycline), aux effets indirects (liés à la perte des symbiotes intestinaux) ; (iii) enfin, dans chapitre 3, nous étendons nos recherches à un troisième niveau trophique: la plante. Nous présentons d'abord un court chapitre sur les défenses chimiques des plantes contre les herbivores, ainsi que leurs mécanismes d'action. Notre étude porte ensuite sur les défenses chimiques du colza (*Brassica rapa*) selon que la plante est attaquée par des larves infectées par *Wolbachia* ou non. Pour cela, nous avons identifié et quantifié les substances volatiles émises par les plantes, ainsi que les taux de glucosinolates contenus dans les tissus.

A la fin de ce manuscrit, nous mettons l'ensemble de nos résultats en perspective dans la discussion générale, en y incluant des données et résultats supplémentaires obtenus au cours de cette thèse.

Chapitre 1 : Influence de *Wolbachia* sur les traits d'histoire de vie de la mouche du chou, *Delia radicum*

Dans cette étude, nous avons étudié l'impact du symbiote intracellulaire *Wolbachia* sur certains traits d'histoire de vie de la mouche du chou *D. radicum*. Pour cela, nous avons préalablement créé deux souches de mouches grâce à des lignées isofemelles, l'une infectée par *Wolbachia*, et l'autre sans le symbiote, à partir d'une souche de laboratoire naturellement infectée à environ 50%. Nous avons ensuite mesuré plusieurs traits d'histoire de vie sur les deux lignées tels que la probabilité de pondre, la fécondité, et la survie des femelles après la ponte ; le taux et la durée d'éclosion ; le taux d'émergence de l'oeuf à l'adulte et le temps de développement total ; le sex-ratio des adultes, leur survie sans nourriture, ainsi que leur taille. Pour la lignée infectée par *Wolbachia*, nous avons également mesuré le taux de transmission verticale du symbiote en analysant via PCR 400 descendants issus de 40 femelles infectées.

La transmission verticale et maternelle de *Wolbachia* était de 100% et nous n'avons trouvé aucune preuve de manipulation de la reproduction telles que l'incompatibilité cytoplasmique, la parthénogenèse thélytoque, la féminisation ou la dégénérescence des embryons mâles. Les effets de *Wolbachia* sur *D. radicum* étaient significatifs pour certains traits, mais modérés, et se compensaient mutuellement (réduction du taux d'éclosion, meilleure survie larvo-nymphale, temps de développement plus long et augmentation de la mortalité des femelles en conditions de stress), ce qui suggère une infection quasi neutre chez cette souche de *D. radicum*.

Chapitre 2 : Effets directs et indirects de la tétracycline sur les traits d'histoire de vie de la mouche du chou (*Delia radicum*)

Pour évaluer les effets du microbiote intestinal sur les traits d'histoire de vie d'un insecte-hôte, de nombreuses études utilisent des antibiotiques à large spectre pour éliminer le microbiote, puis comparent la souche aposymbiotique (= exempte de bactéries) au témoin non traité. Avec ce protocole, les effets observés sont souvent supposés dus uniquement à la perte des bactéries intestinales, et non à un éventuel effet toxique direct

de l'antibiotique, voire à une simple modification de la diversité microbienne (dans le cas où les bactéries ne seraient pas toutes éliminées par le traitement).

Dans cette étude, nous avons essayé de distinguer les effets directs d'un antibiotique (la tétracycline) sur la mouche du chou (effet chimique) de ses effets indirects (dus à la perte partielle ou totale du microbiote intestinal). Nous avons choisi la tétracycline pour cette expérience car il s'agit d'un antibiotique à large spectre, très couramment utilisé dans les études sur les insectes pour éradiquer les symbiotes bactériens. Cependant, en raison de l'origine bactérienne ancestrale des mitochondries, un traitement à la tétracycline peut aussi les impacter négativement, conduisant à une diminution de la production d'ATP chez *Drosophila simulans*. Cela pourrait, de toute évidence, avoir une influence directe sur le phénotype de l'hôte, celle-ci pouvant être aisément confondue avec un effet négatif de la «perte de bactéries». Pour l'expérience qui suit, nous avons utilisé une lignée exempte de *Wolbachia* de façon à ce que les effets éventuellement observés soient uniquement dus soit à l'effet toxique de l'antibiotique, soit à la perturbation du microbiote intestinal. Un protocole couvrant trois générations a été établi, nous permettant de distinguer un effet direct de la tétracycline sur l'hôte d'un effet indirect (perturbation du microbiote).

Nous avons ensuite mesuré six traits "classiques" d'histoire de vie: la probabilité qu'un œuf atteigne l'âge adulte (taux d'émergence), la durée totale de développement, le sex-ratio, la taille adulte, la survie sans nourriture chez des individus vierges, et la charge en œufs matures chez les femelles après 10 jours d'accouplement avec des mâles.

Tous les traits d'histoire de vie, sauf le sex-ratio, ont été affectés par le traitement à la tétracycline. De plus, ces effets pouvaient parfois être détectés jusqu'à deux générations après le traitement. Dans l'ensemble, le taux d'émergence était inférieur, le temps de développement était plus long et la survie était réduite, de même que la charge en œufs des femelles. Les descendants des parents traités à la tétracycline (avec des grands-parents non traités auparavant) étaient les plus significativement touchés, avec trois traits sur cinq sévèrement dégradés (taux d'émergence, survie sans nourriture et charge en œufs). Le seul trait qui a été amélioré par le traitement à la tétracycline était la taille, avec une lignée produisant des individus plus gros lorsque leurs grands-parents avaient été traités, mais pas leurs parents. Nos données suggèrent que l'effet de la perte complète ou partielle du microbiote joue un rôle plus important que tout effet toxique de la

tétracycline elle-même. Elles nous ont permis de conclure également que le microbiote intestinal des descendants semble au moins partiellement hérité de la mère. Dans l'ensemble, cette étude suggère un rôle bénéfique du microbiote intestinal chez cette espèce, car la lignée présentant le meilleur phénotype était, pour chaque trait, celle du traitement témoin.

Chapitre 3 : Influence des symbiotes sur les interactions plantes-insectes

Les insectes sont le plus souvent herbivores, et même si les plantes ne peuvent pas fuir leurs prédateurs phytophages, elles ont tout de même développé de nombreux mécanismes de défense au cours de leur évolution. Au fur et à mesure que les plantes ont mis en place ces mécanismes, les insectes se sont adaptés en retour, en développant une résistance ou tolérance face à ces défenses végétales. Dans le système plante-insecte, nous assistons à une vraie course aux armements.

Lorsqu'un insecte attaque une plante, il laisse de nombreux indices derrière lui que la plante est capable d'identifier spécifiquement de façon à adapter ses défenses. Cette reconnaissance de l'attaque par la plante déclenche une véritable cascade biochimique d'événements, conduisant à de nombreuses modifications physiologiques de la plante. Les phytohormones, molécules de signal endogènes qui contrôlent la physiologie et le développement des plantes, sont également responsables du déclenchement des défenses de la plante (directes ou indirectes) en réponse à l'herbivorie.

Lorsqu'elles sont attaquées, les plantes peuvent augmenter la concentration en composés secondaires de leurs tissus, comme par exemple, les glucosinolates, utilisés dans la défense directe. Ces composés sont stockés sous forme inactive dans les vacuoles des cellules végétales et peuvent être trouvés dans toutes les parties de la plante. Lorsque les tissus de la plante sont endommagés, les glucosinolates contenus dans les tissus sont libérés et entrent en contact avec l'enzyme myrosinase (elle aussi présente dans toute la plante), qui va les hydrolyser et les convertir en divers produits de dégradation toxiques tels que les isothiocyanates, des composés volatils. Suite à une attaque, les plantes peuvent également émettre des mélanges complexes d'autres composés organiques volatils, différents de ceux normalement émis. Ces bouquets d'odeurs défendent la plante

indirectement car ils ne ciblent pas les herbivores mais attirent plutôt leurs ennemis naturels (prédateurs et/ou parasitoïdes).

Pour contrer ces deux mécanismes de défense, les insectes herbivores ont développé plusieurs stratégies. Certains insectes peuvent détourner l'hydrolyse du glucosinolate en nitrile, moins toxique que les isothiocyanates ; tandis que d'autres sont capables de stocker les composés toxiques dans leur hémolymphe, rendant les insectes toxiques à leur tour pour leurs prédateurs. Enfin, certains peuvent également manipuler la biosynthèse des phytohormones végétales pour réduire, voire inhiber complètement, les défenses des plantes, en perturbant les signaux habituels de la plante.

Ces dernières années, de plus en plus d'études ont montré que les symbiotes pouvaient jouer un rôle dans ces interactions plante-insecte, en manipulant directement les défenses des plantes au profit de leurs insectes hôtes. En effet, il a été montré que certains micro-organismes pouvaient interférer directement avec ces défenses en régulant les voies de signalisation des phytohormones.

Delia radicum est un insecte oligophage de la famille des Brassicaceae. Lorsque les larves de cette mouche se nourrissent sur les racines, les taux de glucosinolates et de substances volatiles de la plante sont radicalement modifiés.

Dans cette étude, nous avons cherché à savoir si le symbiote intracellulaire de la mouche du chou, *Wolbachia,* pouvait modifier les réponses du colza (*Brassica napus*) suite à une attaque. Pour ce faire, nous avons déposé sur des racines de colza des larves de *D. radicum* infectées ou non par *Wolbachia*, puis nous nous sommes assurés que l'herbivorie avait bien lieu. Pour cette expérience, nous avons utilisé deux lignées de mouches disponibles au laboratoire, génétiquement similaires, mais avec un statut infectieux différent: une lignée 100% infectée par *Wolbachia* (appelée WP pour *Wolbachia*-plus) et une lignée sans le symbiote (appelée WM pour *Wolbachia*-moins). Après l'attaque des plantes par ces deux types de mouches, nous avons mesuré et comparé les concentrations en glucosinolates et les bouquets de volatils émis entre ces deux traitements.

Une de nos hypothèses était que la réponse des plantes puisse être influencée par *Wolbachia* via la modification de la salive de *D. radicum*. En effet, cette bactérie a déjà été observée dans les cellules des glandes salivaires d'autres diptères, et il est envisageable

que la présence de ce symbiote puisse modifier la composition de la salive. Une seconde hypothèse était que *Wolbachia* puisse modifier indirectement les voies de signalisation des plantes via la modification du microbiote intestinal de son hôte, puisqu'il a déjà été montré que ce symbiote pouvait influencer fortement la diversité des bactéries intestinales. Quel que soit le mécanisme sous-jacent, nous nous attendions dans tous les cas à une modification des réponses de la plante en fonction de la présence/absence de *Wolbachia*.

Dans nos échantillons de plantes, dix glucosinolates différents ont été détectés, dont cinq présents dans les racines et les feuilles, quatre uniquement dans les racines, et un uniquement dans les feuilles. Les niveaux de glucosinolates étaient influencés par le traitement, mais d'une façon différente pour chacun des organes (feuille ou racine).

Les racines attaquées avaient des concentrations en glucosinolates inférieures aux racines témoins, peu importe que la larve possède *Wolbachia* (WP) ou non (WM), excepté pour l'un d'eux, dont la concentration a augmenté. Dans nos échantillons, les concentrations les plus faibles étaient toujours observées dans les plantes attaquées par une larve WP.

Dans les feuilles, la teneur en glucosinolates était similaire entre les plantes témoins et les plantes WM. Cependant, dans les plantes attaquées par les larves WP, les concentrations en glucosinolates ont diminué de façon significative, en particulier pour certains composés aliphatiques (famille de glucosinolates dérivés de la méthionine, isoleucine, leucine ou valine).

Quinze et treize composés volatils ont été détectés dans nos échantillons un jour et trois jours après l'infestation, respectivement, mais les bouquets de composés ne différaient pas significativement les uns des autres selon les traitements.

Le résultat majeur de cette étude est la découverte que *Wolbachia*, symbiote endocellulaire de la mouche du chou, peut en effet influencer la réaction de défense de sa plante hôte. Lors de cette expérience, après que les plantes aient été attaquées avec des larves WP, nous avons observé une diminution des glucosinolates aliphatiques dans les feuilles et, par conséquent, une diminution probable des isothiocyanates volatils associés, ce qui pourrait être avantageux pour *Delia radicum*. En effet, ces composés sont des

signaux olfactifs pouvant être utilisés par les prédateurs de *D. radicum* pour les localiser. De même, une diminution des glucosinolates dans les feuilles est avantageuse pour *D. radicum* car ces composés stimulent la ponte chez cette espèce; cette diminution pourrait donc rendre la plante inattractive pour les femelles conspécifiques, afin d'éviter qu'elles ne pondent leurs oeufs sur la même plante. Cette étude confirme que les interactions plante-insecte sont beaucoup plus complexes qu'il n'y paraît, et souligne l'importance de la prise en compte de chaque niveau trophique (et en particulier de l'influence des micro-organismes de l'insecte ET de la plante) lors de prochaines études.

Discussion générale

Dans cette thèse, nous avons étudié l'holobiote constitué par *D. radicum* et ses symbiotes. Nous avons montré que les bactéries, qu'elles soient situées directement dans les cellules de l'hôte ou dans l'intestin, peuvent avoir des effets variés sur *D. radicum*. Leur influence est d'autant plus grande que les effets d'une perturbation du microbiote peuvent être observés jusque dans les générations futures. De plus, nous avons montré que les symbiotes influencent non seulement leurs insectes hôtes, mais sont également capables de manipuler d'autres niveaux trophiques, tels que les plantes-hôtes de l'insecte.

Quel que soit leur emplacement, les symbiotes bactériens sont donc capables d'influencer de nombreux processus physiologiques et écologiques, à plus d'un niveau trophique (sans parler des interactions probables entre les compartiments cellulaire et intestinal). La plupart des études se concentrent sur les effets d'une catégorie particulière de symbiotes sur leurs hôtes, beaucoup moins analysent les interactions qui peuvent avoir lieu au sein d'un insecte entre ces différents types de micro-organismes, et encore moins prennent en compte le partenaire "plante" en compte. Sachant que la majorité des insectes se nourrissent de plantes et que tous les insectes ont des symbiotes, les interactions possibles semblent illimitées. De plus, d'autres «acteurs» pourraient également jouer un rôle, comme par exemple l'ensemble des bactéries présentes dans l'environnement, avec lesquelles les insectes sont en contact, ce qui pourrait d'autant plus compliquer ces interactions.

Cette thèse démontre qu'il est maintenant nécessaire de prendre en compte tous ces compartiments dans de futures études pour comprendre les possibles relations entre les différents partenaires, écologiques ou évolutives, et leurs éventuelles implications. Même si la prise en compte de ces interactions globales compliquera forcément la tâche, le dialogue cellulaire et moléculaire se déroulant au sein de l'holobiote est cependant à notre portée, grâce aux nombreux outils moléculaires et chimiques à présent disponibles.

Am I simply a vehicle for numerous bacteria that inhabit my microbiome?

Or are they hosting me?

– Timothy Morton –

UNIVERSITE BRETAGNE LOIRE / ECOLOGIE GEOSCIENCES AGRONOMIE ALIMENTATION

UNIVERSITÉ DE RENNES 1

Titre : Impact du microbiote chez un insecte phytophage : interactions entre *Delia radicum* et ses symbiotes intra et extracellulaires

Mots-clés : *Delia radicum*, *Wolbachia*, symbiote, microbiote, antibiotique, traits de vie, *Brassica*, glucosinolates, volatils

Résumé : Les symbiotes d'insectes peuvent considérablement influencer leurs hôtes de diverses manières. Nous avons étudié ici la communauté de microbes de la mouche du chou (*Delia radicum*) et plus particulièrement le rôle de son microbiote intestinal et de *Wolbachia*, une bactérie intracellulaire. La transmission verticale et maternelle de *Wolbachia* était de 100% et nous n'avons trouvé aucune preuve de manipulation de la reproduction telles que l'incompatibilité cytoplasmique, la parthénogenèse thélytoque, la féminisation ou la dégénérescence des embryons mâles. Les effets de *Wolbachia* sur *D. radicum* étaient significatifs mais modérés, et se compensaient mutuellement (réduction du taux d'éclosion, meilleure survie larvo-nymphale, temps de développement plus long et augmentation de la mortalité des femelles en conditions de stress), ce qui suggère une infection quasi neutre chez cette espèce, même si nous avons observé une augmentation de la fréquence d'infection en conditions idéales. L'influence du microbiote intestinal a été étudiée en utilisant un antibiotique, la tétracycline, avec un protocole sur trois générations, ce qui a permis de discerner l'effet direct (toxique) de la tétracycline de ses effets indirects (perte de symbiotes) sur l'hôte. Le traitement antibiotique de *D. radicum* a eu de multiples effets, généralement négatifs, sur les traits d'histoire de vie des descendants, ces effets pouvant être détectés jusqu'à deux générations après le traitement. La perturbation du microbiote intestinal semble avoir un rôle plus important qu'un simple effet toxique de la tétracycline elle-même. De plus, l'étude suggère que le microbiote semble avoir un rôle bénéfique chez cette espèce, et qu'il est au moins partiellement hérité de la mère. Pour finir, nous avons étudié si *Wolbachia* pouvait modifier le dialogue plante-insecte entre *D. radicum* et l'une de ses plantes-hôtes, le colza (*Brassica napus*). La présence du symbiote a diminué les concentrations de glucosinolates dans les feuilles, ce qui suggère que *Wolbachia* pourrait améliorer la fitness de son hôte en diminuant les signaux chimiques de la plante pouvant être utilisés par les conspécifiques et/ou ennemis naturels de *D. radicum*. Cette étude a montré le potentiel d'une bactérie intracellulaire à influencer les relations plantes-insectes et a permis de discuter des interactions tri-trophiques entre les symbiotes, leurs insectes-hôtes et un troisième niveau trophique : la plante. Cette thèse démontre qu'il est maintenant nécessaire de prendre en compte les symbiotes dans de prochaines études, afin de mieux comprendre les relations possibles entre différents partenaires, ainsi que leurs implications écologiques ou évolutives.

Title: Influence of the microbiota on a phytophagous insect: interactions between *Delia radicum* and its intra and extracellular symbionts

Keywords: *Delia radicum*, *Wolbachia*, symbiont, microbiota, antibiotic, life history traits, *Brassica*, glucosinolates, volatiles

Abstract: Microbial symbionts can deeply influence their animal hosts in various ways. Here, we studied the community of microbes of the cabbage root fly (*Delia radicum*) and more precisely the role of its gut microbiota and of *Wolbachia*, an intracellular bacterium. The vertical maternal transmission of *Wolbachia* was perfect, and we found no evidence of manipulation of reproduction such as cytoplasmic incompatibility, thelytokous parthenogenesis, feminization nor male killing. *Wolbachia* infection had significant but moderate and mutually compensating effects on *D. radicum* (reduced hatch rate, improved larvo-nymphal viability, longer development time and increased female mortality in stress conditions), suggesting that infection might be nearly neutral in this strain, although we observed an increase in infection frequency in ideal rearing conditions. The influence of the gut microbiota was studied using an antibiotic, tetracycline, with a protocol spanning three generations, which allowed to discriminate the possible direct (toxic) effect of tetracycline from its indirect effects (due to the loss of gut symbionts). Antibiotic treatment of adults led to multiple and mostly negative effects on life history traits of their offspring and grandchildren. Data suggested a larger role of gut microbiota perturbation than of a toxic effect, that the microbiota was partially inherited maternally, and that the "wild-type" gut microbiota was beneficial in this species. Finally, we investigated whether *Wolbachia* could modify the insect-plant dialogue between *D. radicum* larvae feeding on roots of oilseed rape (*Brassica napus*). The presence of the symbiont decreased glucosinolate concentrations in the leaves, suggesting that *Wolbachia* could increase the fitness of its host by decreasing plant cues used by *D. radicum* conspecifics and/or natural enemies. This study showed the potential of an intracellular bacteria to influence plant-insect relationships, and allowed to discuss the tri-trophic interactions between symbionts, their insect hosts and a third trophic level: the plant. This thesis demonstrates the necessity to consider intracellular and extracellular symbionts in further studies, in order to unravel all the possible relationships between different partners, as well as their ecological or evolutionary implications.