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## The host as an ecosystem

Natacha Kremer

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## Université Claude Bernard - Lyon 1

Ecole doctorale 341 : E2M2  
Evolution, écosystèmes, Microbiologie, Modélisation

### **Habilitation à diriger des recherches (HDR)**

présentée par

**Natacha KREMER**

Chargée de recherche CNRS

## L'HÔTE COMME ÉCOSYSTÈME *THE HOST AS AN ECOSYSTEM*

Soutenue publiquement le 27 novembre 2023, devant le jury composé de :

Christine BRAQUART-VARNIER, Professeure, U. Poitiers

Martin KALTENPOTH, Professor, Max Planck Institute for Chemical Ecology (Jena, Allemagne)

Mathieu SICARD, Professeur, U. Montpellier

Delphine DESTOUMIEUX, Directrice de recherche CNRS, U. Montpellier

Thomas PRADEU, Directeur de recherche CNRS, U. Bordeaux

Claire VALIENTE (UCBL), Maîtresse de conférence, U. Lyon 1

Margaret MCFALL-NGAI, Professor, California Institute of Technology (Pasadena, USA)

Fabrice VAVRE, Directeur de recherche CNRS, U. Lyon 1



## Prolog and acknowledgements

Symbiosis (in the sense of long-term interaction) has always been fascinating to me by its diversity: diversity of symbiotic associations observed in nature, diversity of phenotypes that symbionts can induce on their host, diversity of mechanisms involved in these interactions, diversity in the possible evolution of the symbiotic system... It is a unique opportunity to integrate several levels of study of biological interactions! This may be the reason why I was a bit scattered during my research, studying different models of symbiosis, and addressing various questions at different scales, ranging from the understanding of molecular mechanisms between partners, to the evolution of symbiosis through the study of life history traits.

I have been fortunate to evolve as a scientist in a particularly open-minded environment, with scientists who do not consider things as fixed or categorized, and who try to study scientific questions at different scales and/or using different approaches. I would especially like to express my warmest thanks to my two mentors, Fabrice Vavre and Margaret McFall-Ngai, who passed on to me their passion for research and inspired me with their scientific insight, while giving me their trust and a high degree of freedom when I was a student. It is now my turn to take a step back on the work I have achieved since then, and to define the directions I would like to follow in the future... It took me a while to start writing this piece of work, as I wished my past projects to be finished and published. But I finally realized that science work takes always more time than expected and never ends! I thus decided to write “the state of the art” of where I am now, and to try explaining how I built my research and which difficulties I encountered, rather than to focus only on the results.

I am very grateful to all mutualistic interactions and ecosystems that have developed on this journey. What would research be without teamwork? I would like to thank the students I have (co-)mentored and who have produced most of the results I will present here. It was a pleasure to discuss science and share expertise, but also to learn a lot about what is mentoring. Each interaction was different and very constructive. I would also like to thank all the collaborators in the GEI team, in other teams, in other labs, and the invaluable help of the technical and administrative staff (with a very special mention for H el ene Henri). Together, we have been able to develop exciting scientific projects, set up collaborations, animate scientific networks, organize great conferences or outreach events... Each experience was new and so rewarding! Finally, I would like to thank the amazing scientists who have honored me by being part of my jury. I hope you will enjoy reading this manuscript and I look forward to the discussion we will all have together!



Nadim Karam, sculpture, The Peacock Elephant



# Summary

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# Natacha KREMER

Aim: to provide an evolutionary perspective to the study of symbiosis  
by understanding the molecular interactions between partners

**Chargée de Recherche (CRCN) CNRS since 2014 (sections 27 & 29)**

LBBE (Laboratoire de Biométrie et Biologie Évolutive) | UMR CNRS 5558, Université de Lyon (Lyon 1)  
Department: Multi-scale coevolution | Team: Genetics and Evolution of Interactions

Birthdate: 16/02/1982 | Nationality: French | ■■■■ OrCID #: <https://orcid.org/0000-0001-5678-9416>

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## Education and Professional experience

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- 2010-2014 **Postdoc** | University of Madison, WI, USA.  
Topic: "Establishment and maintenance of the squid/vibrio symbiosis".  
Supervision: M.J. McFall-Ngai (Medical, Microbiology & Immunology department)
- 2005-2009 **PhD** | University Lyon 1, France.  
Topic: "Evolution of dependance in *Wolbachia* symbioses: Study of the genus *Asobara* (Hymenoptera: Braconidae)".  
Supervision: F. Vavre (LBBE).  
+ Teaching (Monitorat) in genetics (64 h/year); University Lyon 1.
- 2003-2005 **Schooling at the École Normale Supérieure** (Paris, concours F/S).  
- Master 2 "Interdisciplinary Approaches to Life" (AIV|ENS, Paris 6, 7 & 11).  
*with honors (mention Félicitations)*  
- Magistère in Biology and Biochemistry (ENS).  
*with honors (mention Bien)*  
Internships (3-6 months):  
- Laboratory of Ecology, ENS Paris / M. van Baalen.  
- Laboratory of molecular, evolutionary and medical genetics, Necker hospital / F. Taddei.  
- Evolutionary Biology Centre, Uppsala (Sweden) / G. Arnqvist, A. Maklakov.  
- Institute of Ecology, Lausanne (Suisse) / L. Keller, M. Chapuisat, B. Holzer.
- 2000-2002 **DEUG Life sciences** at Université Paris 7 (Paris-Diderot).  
*with honors (mention Bien)*
- 1999-2000 **First year of pharmacy** at Université Paris 5 (Paris-Descartes).  
*validated (139/725)*
- 1999 **Scientific baccalaureat**, specializing in physics and chemistry at Lycée Camille Pissarro (Pontoise).  
*with honors (mention Bien)*

## Funding

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### Fellowships

- 2010-13 **Marie-Curie IOF** | FP7-PEOPLE-2010-IOF | 272684 | SymbiOx: 233 k€  
“Role of the oxidative environment in the stability of symbiotic associations”  
Collaborators: M.J. McFall-Ngai (MMI, Madison, WI, USA) and F. Vavre (LBBE, Lyon, France)
- 2006-09 Doctoral scholarship from the French Ministry of Education and Research
- 2002-06 Scholarship to study at the *Ecole Normale Supérieure* (civil servant trainee) from the French Ministry of Education and Research.

### Grants

- 2021-2025 **ANR PRC:** PI. 500k€  
“FBI: Fight Bedbug infestations: Guide insecticide treatments and develop alternative control strategies”  
Collaborators: J. Varaldi (LBBE), A. Zaidman-Rémy (Laboratory of Functional Biology, Insects, Interaction - BF2I) et J.-P. David (Alpine Ecology Laboratory - LECA)  
Topics: WP1 (JV) - Characterization of the diversity and spatial distribution of genetic determinants underlying pyrethroid resistance; development of rapid diagnostic tools to monitor resistance genes in the field.  
WP2 (AZ-R) - Study of the molecular dialogue (dual-RNAseq and metabolomics) between bed bugs and their obligate nutritional symbiont *Wolbachia*.  
WP3 (NK) - Characterization of the interference between *Wolbachia* and insecticides.  
Scope & impact: to improve the targeted use of pesticides and preserve their efficacy (WP1), to develop in the longer-term alternative and complementary methods to insecticides by disrupting the symbiosis (WP2), and to optimize the efficacy of both control methods (WP3).
- 2019-2024 **Pack Ambition recherche Rhône-Alpes** (academic/industry grant): PI. 200 k€. “Symbed: How to break the interactions between bed bugs and their microbiota to control their populations? Development of new methods for targeted control of arthropods to limit the use of insecticides.”  
Collaborators: A. Zaidman-Rémy/A. Heddi (BF2I) and R. Lasseur (IZInnovation company).  
Topics: Setting up of the bedbug farm; general description of the bedbug-*Wolbachia* interaction in a natural symbiotic context (dynamics, RNAseq transcriptomics); functional study of some candidate genes; development of a prototype of a disruption method.  
Scope & impact: developing in the longer-term alternative and complementary methods to insecticides by breaking the symbiosis.
- 2017-2019 **Grant of the ECOFECT LabEx** (Eco-evolutionary dynamics of infectious diseases): Co-PI with François Leulier (IGFL). 170 k€. “ComEndoVir: Interaction between commensal and endosymbiotic bacteria and their impact on viral infection in insects”.  
Topics: Characterization of microbial communities of *Drosophila* larvae trapped in the wild and determination of the influence of *Wolbachia* on this composition; Study of *Wolbachia*-microbiota-virus interactions and their consequences on the host extended phenotype, using the controlled (gnotobiotic) system.  
Scope & impact: better understanding of multipartite interactions and their impact on insect immunity.
- 2016-2020 **ANR JCJC grant:** PI. 310 k€  
“RESIST: Rapid Evolution of Symbiotic Interactions in response to Stress: processes and mechanisms”.  
Topics: Study the evolution of the *Drosophila melanogaster* / *Wolbachia* symbiotic association in response to oxidative stress and viral infection. This project combines the study of evolutionary mechanisms involved in adaptation to stress (within- and between-host selection) and molecular mechanisms (transcriptomics and comparative metabolomics).  
Scope & impact: Ability of vertically transmitted symbioses to respond to environmental stress.

## Organization / facilitation of research

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### Member of steering committees

- 2021- : **REID** (Network of Long-term Interaction Ecology)
- 2019- : **G-RREMI** (Regional Research Group in Microbiology of Interactions)
- 2018- : **Symbiotron** (Secured insectarium platform, University Lyon 1, FR BioEnviS)
- 2023- : **Doctoral School E<sup>2</sup>M<sup>2</sup>** (Evolution, Ecosystems, Microbiology, Modelling)

### Organization of meetings / workshops / prospects

- 2022: International Symbiosis Society & Holobiont meeting (n = 350, main organization board)
- 2022: **Prospective CNRS (INEE)**, presentation and animation of round tables
  - Axis 5: At the border between ecology and evolution
  - Axis 8.4: Health & Environment / the host as an ecosystem
- 2022, 2023: Annual meeting of the **G-RREMI** (n = 70)
- 2022: Annual meeting of the **Santé@LabEx**: forum for LabEx working on health (n = 80)
- 2020/21: **webinars** of the **G-RREMI**
- 2019: Workshop 'Microbiome' (n = 100)
- 2019: Conference of the **LabEx Ecofect**: 'Evolution of infectious diseases: connecting ecological, molecular and computational approaches' (n = 120)
- 2018: Annual meeting of the LabEx **Ecofect** (Eco-evolutionary dynamics of infectious diseases) (n = 70)
- 2017: **Immuninv** meeting (Immunity of invertebrates) (n = 90)
- 2016: **REID** meeting (n = 120)

### Involvement within the laboratory

- 2021-: Representative of the 'cadres B' at the UMR council
- 2020-: Implementation of the electronic laboratory notebook (ElabFTW)
- 2018-: Participation in working groups (environmental footprint, quality of life at work)

## Scientific expertise

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### Expertise for ANSES - Ministry of Ecological Transition and Ministry of Solidarity and Health

2022- Expert *intuitu personae* for the "bed bugs" working group (Orientation towards bed bug prevention and control)

### Edition / Expertise of publications and research projects

**Research topic** in Frontiers in Cellular and Infection Microbiology - Parasite and Host (2021)

**Review editor** for Frontiers in Microbiology – Frontiers in microbial symbioses (2014-)

**Recommender** for Peer Community in (PCI) Evolutionary Biology (2017-), PCI Zoology (2020-)

### Expertise for scientific journals:

Antioxidant and Redox Signaling, Behavioral genetics, BMC Microbiology, Comparative Biochemistry & Physiology, FEMS Microbiology Ecology, FEMS microbiology Letters, Gene, Genome Biology & Evolution, Insect Science, Insect biochemistry & molecular biology, J. of Animal Ecology, J. of Entomological Research, J. of Insect physiology, Heredity, ISME journal, mBio, Microbiology Open, Molecular Ecology, Nucleic Acid Research, Parasite & vectors, PCI Zoology, Peer J, PLoS One, PLoS Neglected Tropical Diseases, PNAS, Proc. of the R. Soc. B, Scientific reports.

### Expertise for funding agencies:

Junior project proposal of the Czech Science Foundation (2016), Dim-1Health (ANSES - 2017), ERC Starting grant (2018), Anti-VeC Network / pump-priming funding (2018)

## Expertise in evaluation committees

Member (examiner) in thesis jury of:

- **Jeff Rouil**: 'Evolution and dynamics of the di-symbiotic system in aphids of the genus *Cinara*', CBGP Montpellier (2023).
- **Marie Buysse**: 'Inheritable endosymbiotic systems in ticks'. MIVEGEC, Montpellier (2022)
- **Maurine Hammel**: 'Biology, ecology and evolution of MtrBTN2 transmissible mussel cancer *Mytilus* sp.', IHPE & ISEM, Montpellier (2021).
- **Corentin Sochard**: 'The impact of microbial partners on insect interactions with their biotic environment: a behavioral approach to symbiotic associations', IGEPP, Rennes (2020).
- **Antoine Rombaut**: 'Understanding and controlling the damage caused by the pest *Drosophila suzukii* in the vine agrosystem', CBGP, Montpellier (2019).
- **Bérénice Piquet**: 'The flexibility of symbioses between bivalves and chemotrophic bacteria: mechanisms, regulation and resilience', UMR ADMM, UPMC/Roscoff (2018).
- **Aude Lucasson**: 'Characterization and diversity of mortality syndrome mechanisms affecting juvenile mussels *Crassostrea gigas*', UMR 5244 IHPE, Montpellier (2018).
- **Mine Altini**: 'Ecology and Evolution of *Culex pipiens-Wolbachia-Densovirus* Tripartite Interactions', ISEM, Montpellier (2018).
- **Justin Maire**: 'Immune and developmental regulations in host-symbiont interactions in the cereal weevil *Sitophilus spp.*', BF2i INSA Lyon (2018).
- **Romain Pigeault**: 'Evolutionary ecology of host/mosquito/Plasmodium interactions: a source of heterogeneity in vector infection', MIVEGEC, Montpellier (2015).
- **Audrey Vanhove**: 'Intracellular survival, cytopathic effects and virulence of *Vibrio tasmaniensis* LGP32, an oyster pathogen *Crassostrea gigas*', IHPE, Montpellier (2014).

Participation in selection committees

- 2017 (maître de conférence (lecturer) - position 4423).  
UMR 5557, Laboratoire d'écologie microbienne, Claire Valiente-Moro's team.  
Topic: Microbial ecology of microbiota-mosquito interactions.

## Mentoring

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Undergraduates: 9

- **Justine Bisson** (L3 ENS Lyon; 2008; 2 months; co-mentoring with F. Vavre)  
→ Topic: Polymorphism of *Wolbachia* dependence phenotype in *Asobara tabida* populations.
- **Ami Yamamoto** (L1-L2 UW Madison; 2010-2012; 1.5 years)  
→ Topic: Influence of iron in the squid-vibrio symbiosis.
- **Lawrence Zhou** (L1-L2 UW-Madison; 2012-2014; 2 years)  
→ Topic: Influence of oxidative stress in the establishment and maintenance of the squid/vibrio symbiosis  
📖 Publications linked to the project: Kremer et al., Procs R. Soc. B 2014; Schwartzman et al., PNAS 2015
- **Manon Villa** (L3 Montpellier; 2015; 2 months, co-mentoring with D. Monnin)  
→ Topic: Influence of oxidative stress on symbiont density and life history traits of various *Drosophila simulans* / *Wolbachia* associations.  
📖 Publications linked to the project: Monnin et al., PCJ Evol. Biol. 2020
- **Marine Vallat** (DUT Biological Engineering Lyon1; 2017; 3 months; co-mentoring with V. Raquin)  
→ Topic: Development of PCR-RFLP to identify different species of *Drosophila* larvae.  
📖 Publications linked to the project: Raquin et al., Ecology & Evolution 2018
- **Antoine Deydier** (L3 Lyon1; 2018; 2 months; co-mentoring with F. Vavre and Y. Voituron)  
→ Topic: Evaluation of the respiratory capacity of *Wolbachia*.
- **Garance Donzé** (L3 ENS Lyon; 2019; 2 months; co-mentoring with A. Bénard)  
→ Topic: Characterization of the intensity of symbiotic bottlenecks.

- **Célia Novo** (L3 Lyon1; 2019; 2 months; co-mentoring with A. Bénard)  
→ *Topic*: Evolution of viral and bacterial intra-host dynamics in the *Drosophila melanogaster* / *Wolbachia* / DCV model system.
- **Lou Guyot** (L3 ENS Lyon; 2022; 2 months)  
→ *Topic*: Variability of resistance to DCV in different populations of *Drosophila melanogaster*.

#### Master 2 students: 5

- **Caroline Michaud** (Poitiers; 2015; co-mentoring (40%) with D. Monnin (40%) and F. Vavre (20%))  
→ *Topic*: Study of the heterogeneity and evolution of symbiotic populations in the association between *Drosophila melanogaster* and the *Wolbachia* strain wMelPop.  
📖 *Publications linked to the project*: Monnin et al., PCJ Evol Biol 2020  
🕒 *Student's future*: PhD with Franck Dedeine, University of Tours; Post-doc ANSES
- **Camille Huot** (Poitiers; 2016; co-mentoring (70%) with F. Vavre (30%))  
→ *Topic*: Oxidative stress response in *Drosophila/Wolbachia* symbiotic associations and consequences on antiviral protection.  
🕒 *Student's future*: PhD with Eve Toulza and David Duval, University of Perpignan; Science teacher
- **David Barrau** (Lyon 1; 2017; co-mentoring (30%) with G. Marais (40%) and F. Vavre (30%))  
→ *Topic*: Origin and consequences of asexuality in insect-*Wolbachia* symbioses.  
🕒 *Student's future*: Reorientation (permaculture)
- **Alexis Bénard** (Nice; 2017; co-mentoring (80%) with F. Vavre (20%))  
→ *Topic*: Characterization of the density regulation determinisms of *Wolbachia* in *D. melanogaster*.  
📖 *Publications linked to the project*: Bénard et al., PCJ Evol Biol 2020  
🕒 *Student's future*: PhD with N. Kremer and F. Vavre
- **Imane Belkacemi** (Lyon 1; 2019; co-mentoring with S. Bourg (40%) and E. Rajon (30%))  
→ *Topic*: Comparison of life history trait relationships in three *Drosophila* species.  
🕒 *Student's future*: Reorientation (catering)

#### Assistant engineers: 3

- **Angelo Jacquet**: RESIST project, ecology & molecular biology (15 months; 2017-2019)
- **Cécile Jacot-des-combes**: RESIST project, molecular biology (4 months; 2021)
- **Elodie Rosinski**: Bedbug rearing and optimization (42 months; 2022- )

#### PhD students: 5 (+1)

- **David Monnin** (2013-2016; co-supervision (30%) with F. Vavre (40%) and E. Desouhant (30%))  
→ *Topic*: Oxidative homeostasis and evolution of dependence in insect / *Wolbachia* symbioses.  
📖 *Publications linked to the project* (5): Moné et al. Procs R Soc B 2014; Monnin et al. JEB 2016; Lopez-Maestre et al., NAR 2016; Monnin et al., PloS One 2017; Monnin et al., PCJ Evol Biol 2020.  
🕒 *Student's future*: Post-doc with M. Shapira (UC Berkeley); Post-doc with Henry Lee (Queen Mary University of London)
- **Salomé Bourg** (2013-2016; co-supervision (20%) with E. Rajon (50%) and F. Menu (30%))  
→ *Topic*: The evolution of mechanism underlying the allocation of resources and consequences on the shape of trade-offs in multicellular organisms.  
📖 *Publications linked to the project*: Axis of her thesis not valorized yet in a publication.  
🕒 *Student's future*: Post-doc at the Norwegian University of Science and Technology
- **Alexis Bénard** (2013-2016; co-supervision (80%) with F. Vavre (20%))  
→ *Topic*: Influence of environmental stressors on the *Wolbachia-Drosophila melanogaster* association.  
📖 *Publications linked to the project* (2 (+2)): Bénard et al., Frontiers in Ecology and Evolution 2020; Bénard et al. PCJ Evol Biol 2020; *Bénard et al., in prep* (x2)  
🕒 *Student's future*: reorientation (scientific escape games)

- **Marius Poulain** (2020-; co-supervision (40%) with A. Zaidman-Rémy (40%) and F. Vavre (20%))  
→ *Topic*: Study of the interaction between the bedbug and its obligatory nutritional symbiont, *Wolbachia*.  
📖 *Publication linked to the project*: Poulain et al., in prep (x2)
- **Camille Mayeux** (2020-; close collaboration on topics linked to viral infections)  
→ *Topic*: Control of transposable elements and response to viral infections in *Drosophila*.  
📖 *Publication linked to the project*: Mayeux et al., in prep (x2)
- **Raphaël Jorge** (2022- ; co-supervision (40%) with A. Zaidman-Rémy (40%) and F. Vavre (20%))  
→ *Topic*: Study of the mechanisms involved in the dependence between bedbugs and their nutritional symbionts.

#### Post-doctorant: 1

- **Vincent Raquin** (2017-2019; co-supervision with F. Leulier (50%))  
→ *Topic*: Interaction between commensal and endosymbiotic bacteria and their impact on viral infection in insects.  
📖 *Publications linked to the project* (1 (+2)): Raquin et al. Ecology & Evolution 2018; Raquin et al. in prep (x2)  
🕒 *Student's future*: Post-doc IVPC/LEM; Associate professor EPHE (IVPC)

#### Member of the steering thesis committees: 7

- Pierre Antonelli (Microbial Ecology Laboratory (LEM), Lyon – tutor)
- Lena Klay (iEES Paris / Camille Jordan Institute)
- Estelle Chabanol (Pasteur Institute Guyane)
- Mélanie Gasser (Microbial Ecology Laboratory (LEM), Lyon)
- Romain Guillot (Biology Center for Population Management (CBGP), Montpellier)
- Aude Lucasson (Host-Pathogen-Environment Interaction Laboratory (IHPE), Montpellier)
- Bérénice Piquet (UMR Adaptation and diversity in the marine environment, UPMC/Roscoff)

## Scientific production

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### Synopsis

#### Publications:

- 31 publications, among which
  - 25 in peer-reviewed journals
  - 4 peer-reviews
  - 1 congress report
  - 2 commentaries
- 3 focus / book chapters
- 2 participations to magazines

#### Oral communications:

- 9 invited conferences
- 7 invited seminars
- 13 oral communications in international meetings
- 18 oral communications in national meetings
- 17 posters

Journal	Impact Factor 2022	Article (publication year)	Review / commentary (year)
Cell Host & Microbe	31,31	2013	
Nucleic Acid Research	19,16	2017	
PNAS	12,53	2015, 2019	2013
eLife	8,71	2014	
mBio	7,87	2012	
PLoS Pathogens	6,82	2009	
Molecular Ecology	6,62		2011
Communications Biology	6,55	2021	
mSystems	6,5	2018	
Proceedings of the Royal Society Series B	5,53	2005, 2014	2014
Environmental microbiology	5,49	2013	
Current Opinion in Insect Science	4,58		2014
Frontiers in Ecology & Evolution	4,17		2020
Evolution	4,17	2010	
Heredity	3,82	2009	
Biology Letters	3,81	2006	
PLoS One	3,75	2017	
BMC Microbiogy	3,61	2012	
Microbiology Open	3,14	2018	
Ecology & Evolution	2,91	2018	
Behavioral Ecology	2,67	2007	
Naturwissenschaften	2,43	2011	
Journal of Evolutionary Biology	2,41	2006, 2016	
Entomological Research (congress report)	1,36		2007
Peer Community Journal (Evolutionary Biology)	In progress	2020, 2021	2017

legend: first author, last author

Publications in peer-reviewed journals: 31

H-index = 20;  $i_{10}$ -index = 26;  $i_{40}$ -index = 16; mean = 128 citations / publication (source: google scholar)

(S: PhD student/post-doc under my (co-)supervision; T: article published during my PhD; P: article related to my post-doc; □: review/commentary)

- S| Bénard A, Henri H, Noûs C, Vavre F, Kremer N. 2021. *Wolbachia* load variation in *Drosophila* is more likely caused by drift than by host genetic factors. **Peer Community Journal, section Evolutionary Biology** | <https://doi.org/10.24072/pcjournal.50>.
- Gauthier J, Boulain H, van Vugt JJFA, Baudry L, Persyn E, Aury J-M, Noel B, Bretaudeau A, Legeai F, Warris S, Chebbi MA, Dubreuil G, Duvic B, Kremer N, et al. (x 34), Huguet E, Herniou EA, Drezen JM. 2021. Chromosomal resolution reveals symbiotic virus colonization of parasitic wasps genomes. **Communications Biology**. 4(104).
- S| Monnin D, Kremer N, Michaud C, Villa M, Henri H, Desouhant E, Vavre F. 2021. Experimental evolution of virulence and associated traits in a *Drosophila melanogaster* – *Wolbachia* symbiosis. **Peer Community Journal section Evolutionary Biology** | <https://doi.org/10.24072/pcjournal.9>.
- S| Bénard A, Vavre F, Kremer N. 2020. Stress & Symbiosis: heads or tails? **Frontiers in Ecology and Evolution**. 8:167.

- P| Belcaid M, Casaburi G, McAnulty SJ, Schmidbaur H, Suria AM, Moriano-Gutierrez S, Pankey SM, Oakley TH, [Kremer N](#), Koch EJ, Collins AJ, Nguyen HLS, Goncharenko-Foster I, Minx P, Sodergren, E, Weinstock G, Rokhsar DS, McFall-Ngai MJ, Simakov O, Foster JS, Nyholm SV. 2019. Symbiotic organs shaped by distinct modes of genome evolution in cephalopods. *PNAS*. 116(8):3030-35.
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#### Book chapters: 3

- « Et la lumière fut... comment la sépiole recrute des symbiotes luminescents ». In '**101 secrets du vivant, l'ADN des écosystèmes**' (2018). CNRS/Cherche-midi. Dir: D. Faure, D. Joly, S. Salamitou.
- Focus "Petite seiche d'Hawaï cherche bactérie pour l'éclairer" in the chapter 'Lumière sur des couples fusionnels : les microbes et leurs hôtes'. In "**Empreinte du vivant, l'ADN de l'environnement**" (2015). CNRS/Cherche midi. Direction : D. Joly, D. Faure, S. Salamitou.
- "Is symbiosis evolution influenced by the pleiotropic role of programmed cell death in immunity and development?". Vavre F, Kremer N, Pannebakker B, Loppin B, Mavingui P (2008). In: **Insect Symbiosis** vol. 3. Eds K. Bourtzis & T.A. Miller, CRC Press, Boca Raton. pp. 57-77.

#### Forthcoming submissions and Publications in preparation: 2+6

- Poulain M, Rosinski E, Henri H, Balmand S, Delignette-Muller ML, Heddi A, Lasseur R, Vavre F, Zaidman-Rémy A, Kremer N. Development, feeding and sex shape the relative quantity of *Wolbachia*, the nutritional obligatory symbiont of bed bugs. *Forthcoming submission to Frontiers in Ecology and Evolution*, special issue on *Wolbachia*.
- Mayeux CA, Larue A, Oliveira DS, Henri H, Rebollo R, Kremer N, Chambeyron S, Fablet M. Transposable elements are major players of the arms race between a virus and its *Drosophila* host. *Forthcoming submission to Science*.

- **Raquin V**, Galvão Ferrarini M, Saint-Michel E, Hughes S, Gillet B, Leulier F, **Kremer N**. Polymicrobial association promotes larval performance upon oral exposure to viral pathogen in an experimental *Drosophila* holobiont model. *In preparation for ISME*
- **Bénard A, Jacquet A**, Guyot L, Henri H, Vavre F, **Kremer N**. Impact of oxidative stress and viral infection on the *Drosophila melanogaster* / *Wolbachia* symbiosis and its evolution. *In preparation*.
- **Mayeux CA**, Larue A, Garambois C, Henri H, Rebollo R, **Kremer N**, Chambeyron S, Fablet M. Transposable elements are modulated by various types of virus in *Drosophila melanogaster* and *Drosophila simulans*. *In preparation*.
- **Poulain M**, Balmand S, **Rosinski E**, Heddi A, Lasseur R, Vavre F, Zaidman-Rémy A\*, Kremer N\*. Infection dynamics of *Wolbachia* in bedbug ovaries. *In preparation*.
- **Raquin V**, Henri H, Minard G, Leulier F, **Kremer N**. Influence of diet, trapping site and *Wolbachia* on bacterial and fungal communities of fruit fly larvae. *In preparation*.
- **Bénard A, Jacquet A**, Henri H, El Filali A, Galvão Ferrarini M, Vavre F, **Kremer N**. Transcriptomic and metabolomic impact of Paraquat ingestion in the symbiosis between *Drosophila melanogaster* and *Wolbachia*. *In preparation*.

#### Scientific advice for press release in magazines: 2

- “Une association lumineuse”. *Entr’espèces*. Bruno Corbara. n°14 (2015), p72-74
- “Comment une seiche lilliputienne éclaire la recherche ?” *Science et avenir* (2015) Rachel Mulo.

#### Invited conferences: 9

- MEEDIN (Montpellier Ecology and Evolution of Disease Network) webinar (2023 – online)
- 26<sup>th</sup> European Meeting of PhD Students in Evolutionary Biology EMPSEB (2021 – online)
- GDR “Complex transcriptomics”
- Young investigator research day “Function & origin of metaorganisms” (2017 - Kiel, Germany)
- Jacques Monod Conference: “Open Questions in Disease Ecology and Evolution: from Basic Research to Evolutionary Medicine” (2017 - Roscoff, France)
- Meeting “Ecology & Behaviour” (2016 - Lyon, France)
- Federation of European Biochemical Societies (2015 - Berlin, Germany).
- ESF/EMBO “integrated insect immunology” (2013 - Pultusk, Poland)
- Gordon conference “Bacterial adhesion and signal transduction” (2011 - Newport, RI, USA)

#### Invited seminars: 7

- B. Rader (2023 – Southern Illinois University (USA) – online)
- A. Vigneron/M. Kaltenpoth (2021 – U. Mainz (Germany) – online)
- G. Mitta (2016 - U. Perpignan)
- T. Bosch (2010, 2016 – U. Kiel (Germany))
- Microbes in Health & Diseases (2010 – Madison (USA))
- F. Melzner (2010 - IFM Geomar (Germany))

#### International meetings (oral): 11 | +2 presented by students

- International Symbiosis Society (2022, 2012)
- Jacques Monod conference “Integrated insect immunology: controlling infections” (2019)
- Royal entomological Society Insect (2018)
- European Society for Evolutionary Biology (2015, 2018)
- *Congress of Entomology* (2014)
- Squid-vibrio meeting (2010, 2011, 2013)
- American Society for Microbiology (2011)

- *Wolbachia* conference (2008)
- Workshop “cell death in development and aging” (2007)

**N**ational meetings (oral): 13 |+5 *presented by students*

- Network “Network of Long-term Interaction Ecology” (REID): x4 (+2)
- Immunity of Invertebrates (Immuninv): x5
- French *Drosophila* meeting
- Workshop “Evolution of infectious diseases: connecting ecological, molecular and computational approaches”: x1 (+1)
- “*Petit Pois Déridé*” (Population genetics national meeting)
- “*Journée des entomophagistes*” (meeting on parasitoids)
- *Annual meeting of the Ecofect LabEx*
- *Santé@labex*

**P**osters: 6 |+11 *presented by students*

- European Society for Evolutionary Biology (ESEB): x2
- Jacques Monod conference
- Winter school: “Concepts and methods in ecological genomics of coevolutionary interactions: bridging the gap between theory and empirical studies”
- “Immunity and Symbiosis” workshop
- French *Drosophila* meeting
- *International Symbiosis Society*
- *Wolbachia* conference
- *Regional workshop “Microbiome”*
- *Annual meeting of the regional research group in interaction microbiology (G-RREMI).*

## Main collaborations

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- Greg Hurst | University Liverpool (UK), Evolution, Ecology & Behaviour
- Oliver Otti | University of Dresden (Germany), Applied Zoology
- Stefanos Siozios | University Liverpool (UK), Evolution, Ecology & Behaviour
- Nicolas Rode | Montpellier, Centre de Biologie pour la Gestion des Populations
- Anna-Zaidman Rémy | INSA Lyon, Laboratoire Biologie Fonctionnelle, Insectes, Interactions
- Aziz Heddi | INSA Lyon, Laboratoire Biologie Fonctionnelle, Insectes, Interactions
- François Leulier | Institut de Génomique Fonctionnelle de Lyon
- Fabrice Vavre | UCBL, Laboratoire de Biométrie et Biologie Evolutive
- Marie Fablet | UCBL, Laboratoire de Biométrie et Biologie Evolutive
- Julien Varaldi | UCBL, Laboratoire de Biométrie et Biologie Evolutive

## Teaching

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**M**onitorat: total 64h eq TD/year for 3 years between 2016 et 2019: L1, L2, L3 – UCBL

- Mendelian genetics (Practical courses: TP)
- Non-Mendelian genetics (Tutorials: TD)
- Population genetics (TD, TP)
- Molecular genetics (TP)

## Teaching since recruitment at CNRS:

- Symbiosis (Lecture)
  - Master 2 *Biology Ecology Evolution* in Poitiers (3h/year since 2015)
  - Master 2 *Integrative biology of interactions* in Montpellier/Perpignan (2h/year since 2021)
- Population genetics (L3, TP, 2h/year: 2015, 2019)
- Molecular genetics (L2, TP, 6h/year: 2015, 2016, 2020)

## Outreach

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- « **Fête de la science** » (2023, 2017), LBBE/CNRS Villeurbanne
- **Theater play** (2022): Participation in the creation of a play from the n<sup>th</sup> company (presentation of my research projects with the director Claire Truche: "*Insectes : histoire en (dés)équilibre*" (+ exchange with the public))
- **Pint of Science** (2022): "*Symbiose : des colocataires aux fonctions étonnantes! Des symbiotes pour contrôler la transmission d'agents pathogènes par les moustiques : jouons-nous avec le feu ?*"
- **Street Science** (2021, 2022): "*La symbiose dans tous ses états*" (elementary school, families)
- **Declics** (2021, 2022): Dialogues between researchers and high school students.
- **Université ouverte** (2020-2021), Lyon 1. Conference "*Qui sommes-je ? Qui suis-nous ? Conflit & coopération en évolution*" : "*Jouons collectif ! La coopération, un moteur de l'évolution*"
- '**Mercredis de Lyon 1 Sciences**' (2018): hosting high school students at the university
- **Science en marche** (2014) Lyon – scientific animation, 'LudoViro' game
- 2<sup>e</sup> **French-American Science Festival** "*Sharing Experiences, for a New Insight on Sustainable Development*" Chicago, IL (2012).
- **Darwin day**, Wisconsin Institute of Discovery, Madison, WI.
  - The mating game (2013)
  - Unnatural history (2012)
  - Know Thyself. Humans & Evolution (2011)
- Fondation "**La Main à la Pâte**" for science education (2004). 8-year old kids.

## On-going training

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- 2023: Creativity, synthesis and presentation: the advantages of **mind maps** (Urfist)
- 2022: **Fire safety**
- 2021: Control and operation of an **autoclave** in a laboratory and pharmaceutical environment (LBBE - ESMM)
- 2021: Automatic reporting with **Rmarkdown** (Urfist)
- 2019: Basics of **time management** (CNRS, Lyon)
- 2019: **Statistics**: multivariate analyses, linear model, mixed model, longitudinal data (LBBE, VetAgrosup)
- 2018: MOOC **Metabolomics** (FunMooc)
- 2017: **First aid** at work (French Red Cross, Lyon)
- 2017: **Management** - Leading a team of researchers (INSERM/CNRS, IFSeM)
- 2017: **High-throughput sequencing**: non-model organisms (LBBE, CNRS)
- 2017: Thematic school "Concepts and methods in ecological genomics of **coevolutionary interactions**: bridging the gap between theory and empirical studies" (Weggis, Switzerland)
- 2016: Advanced use of a **confocal microscope** (Zeiss, Lyon)
- 2015: Expert **annotation of insect genomes** (INRAE Rennes/Tours)
- 2007: **In situ hybridization** (CTμ Lyon)

# PAST RESEARCH PROJECTS



# Introduction

## Symbiosis and dependence in a nutshell

Symbiotic interactions, which include the whole continuum between parasitism and mutualism, are omnipresent in nature. They involve a wide variety of organisms (*i.e.*, bacteria, virus, fungi, prokaryotes, small eukaryotes, ...) which can be associated with their host in a more or less intimate way (*i.e.*, ectosymbiont, extracellular or intracellular endosymbiont). The balance between the **cost** associated to the presence of the symbiont (*e.g.*, use of nutrients and cellular machinery, virulence) and its **benefit** (*e.g.*, provision of nutrients, immune protection against pathogens) defines the position of the association along the continuum between parasitism (cost > benefit) and mutualism (cost < benefit). Another key parameter in symbiotic associations is the level of **dependence** between partners. While dependence generally results from a long coevolutionary process in mutualistic associations, it can also rapidly evolve in parasitic associations (de Mazancourt *et al.* 2005; Chomicki *et al.* 2020; Hammer 2023). The positions along the continuum and in term of level of dependence between partners are thus not static and can evolve over time and in response to environmental conditions (Drew *et al.* 2021).

An important feature of these symbioses is their **transmission mode** (Moran *et al.* 2008; Alizon *et al.* 2009, 2013; Sachs *et al.* 2011): we can distinguish symbionts that are vertically transmitted from those that are horizontally transmitted. In vertically transmitted symbioses, symbionts are inherited from mother to offspring, and are generally present in the eggs, which ensures a “fidelity” of partners. The global alignment of interests between the host and the symbionts tends to favor mutualistic interactions, even if cases of parasitic interactions are described, mainly due to a conflict of transmission between host and symbiotic genes. In horizontally transmitted symbioses, symbionts are acquired from the environment and colonize the host anew each generation. As the interests of the host and the symbionts are less aligned, this transmission mode generally favors the evolution of virulence, with a trade-off between transmission and virulence. Nevertheless, cases of mutualism can be observed, especially when there is a selection of the symbiotic partner by the host during the colonization process, and possibly a sanction of the cheaters.

## Evolution of the concept of symbiosis and of its study

Symbiotic interactions are classically studied in a binary manner, which allows to describe the impact of the symbiont on host life history traits, and reversely the influence of the host on symbiont population dynamics. The study of binary associations also makes possible to finely characterize the mechanisms involved in the dialogue between partners (*e.g.*, colonization, infection/proliferation, provision of a benefit, immune response, control).

Studies started more recently to take into account the diversity of host and microbial genotypes, which helps to understand the competition between strains within a host and between hosts, the evolution of resistance and tolerance, or the mechanisms involved in arms race between partners...

A new paradigm now tends to consider the host as an “holobiont”, or even an “ecosystem”. The concept of **holobiont** has been introduced by Adolf Meyer-Abich in 1943 (Meyer-Abich 1943; Baedke *et al.* 2020), and will be considered here in its broadest definition, as the individual host and its

symbiotic community<sup>1</sup>. The definition of **ecosystem** was given in 1953 by Howard T. Odum, as “the largest functional unit in ecology, since it includes both living organisms and the abiotic (*i.e.*, non-living) environment, each influencing the properties of the other” (Odum 1953). This ecosystemic view implies to study each symbiont as a potential modifier of the host's extended phenotype, but also to consider the role of symbiotic interactions in the expression of this extended phenotype, and the influence of the environment on the system. Communities can be investigated within a host (*e.g.*, gut microbiota) to study multipartite interactions such as competition, facilitation, or exclusion. Interestingly, concepts of community ecology can also be applied to address questions of establishment, stability or dysbiosis, and the evolution of such communities can be studied in response to environmental changes or various selective pressures.

## Outline

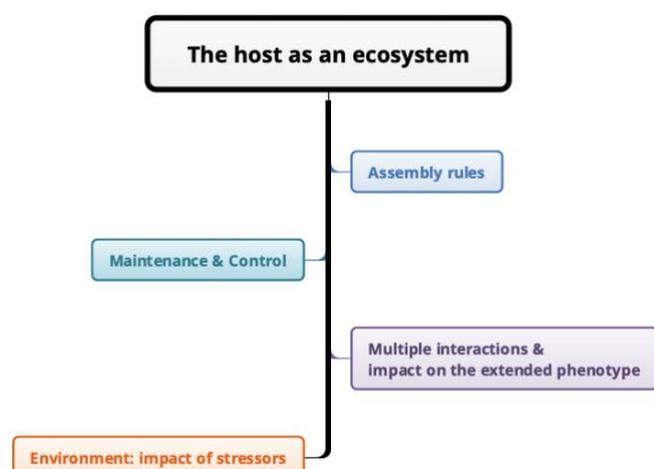
My research work fits into this conceptual framework, as I took advantage of the diversity and complexity of symbiotic associations (binary or multiple) to question **the host as an ecosystem**, using different approaches. In this manuscript, I will thus present different aspects of the functioning of a “symbiotic ecosystem”, based on my past research projects:

(i) focusing on horizontally transmitted symbioses, I will first describe some **assembly rules** between partners of the ecosystem. In particular, I will describe environmental factors that can shape a gut community in flies, but also host mechanisms that filter the surrounding community to select a unique bacterial partner.

(ii) While an ecosystem is dynamic, it also shows a certain stability. In established symbioses, the questions of **maintenance** and **control** of the symbiotic partners are central, and I will present examples in horizontally and vertically transmitted symbioses.

(iii) An ecosystem is a network of interacting species that can modify the niche. I will here describe how **interactions between multiple symbionts** within a host can impact the host extended phenotype.

(iv) Finally, the resilience of an ecosystem to **environmental stressors** is a key parameter to determine its evolution in changing environment. Symbiosis can provide a benefit to its host in buffering the stress, but can also be its Achilles' heel when hosts rely on symbionts for survival. After presenting the impact of acute stressors on the symbiotic association, I will present results of experimental evolution in fruit flies, and nuance the role of vertically transmitted symbioses in adaptation.



After discussing these various points in a more integrative way, I will end by presenting my current research projects / involvements, as well as the directions I would like to develop in the future.

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<sup>1</sup> The concepts ensued from « holobiont » are diverse and controversial. They will be debated in depth in the discussion.

## Models of symbiosis

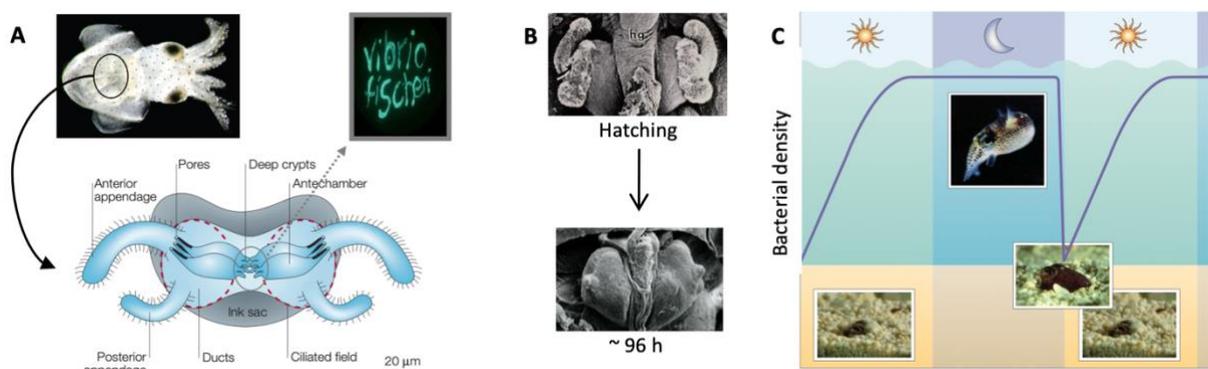
I have chosen to present my past research activities by scientific questions, which implies some “*saucissonnage*”<sup>2</sup> of the study models. Before I start presenting these results, I thus need to introduce the basic characteristics of the different model systems I worked with, to show how they are relevant to the questions raised above.

### The squid-vibrio symbiosis

*Euprymna scolopes* is a bobtail squid, which is always associated with the bioluminescent bacterium *Vibrio fischeri* in its natural environment. Within the squid, *V. fischeri* settles in a specific organ (the light organ) and regulates by quorum sensing the emission of light (via the *Lux* operon). This extracellular bacterium is horizontally transmitted to the squid, which hatches aposymbiotically<sup>3</sup>. Interestingly, *V. fischeri* represents less than 0.1% of the bacterioplankton population, but is the only bacterial genus able to efficiently colonize the light organ anew each generation. The establishment of the symbiosis is associated with morphological changes of the light organ, mostly a regression of the ciliated appendages that recruit *Vibrio fischeri*. Another important characteristic of this symbiotic system is that 95% of the bacterial population is vented<sup>4</sup> out of the light organ in the surrounding sea water, each dawn, and enriches the sea water in *V. fischeri*. The population remaining in the crypts of the light organ proliferates during the day and reaches its maximum density at night

Figure 1; for more details, see reviews Nyholm & McFall-Ngai 2004, 2021) .

→ Because of the specificity of transmission and regulation, the accessibility of the light-organ epithelium and the binary nature of the association, it makes a model of choice to study the mechanisms underlying the establishment and the maintenance of the association in a horizontally transmitted symbiosis.



**Figure 1 | Main characteristics of the *Euprymna scolopes* / *Vibrio fischeri* model system.**

**A.** Detail of the luminous organ at hatching. After aggregating at the cilia, the bacteria migrate towards the pores to reach the crypts. **B.** Developmental changes in the luminous organ due to the establishment of symbiosis. **C.** Variation in bacterial density in crypts during the adult day/night cycle.

Modified from (Nyholm & McFall-Ngai 2004).

<sup>2</sup> splitting

<sup>3</sup> without symbionts in the light organ

<sup>4</sup> Expelled out of the light organ, like a ‘toothbrush’



In the fruit fly *Drosophila melanogaster*:

*Drosophila melanogaster* constitutes a model system to study *Wolbachia* (Serga *et al.* 2023). We have chosen to study two strains in particular:

- The **wMelCS** strain is a natural strain of *Drosophila melanogaster*, present at moderate prevalence in field populations (Versace *et al.* 2014), and currently being replaced by a phylogenetic close strain wMel (Riegler *et al.* 2005). They infect various fly tissues at low density (Dobson *et al.* 1999), but the relative quantity of wMelCS is higher compared to that of wMel (Chrostek *et al.* 2013). These strains induce a variable level of cytoplasmic incompatibility (see review in Merçot & Charlat 2004), and protect flies in presence of RNA positive viruses such as the *Drosophila C virus* (Teixeira *et al.* 2008), proportionally to their density within the fly (Chrostek *et al.* 2013).

→ *These strains are relevant to study infection mechanisms in a natural context of symbiosis*

- The **wMelPop** strain, whose origin (natural or lab (mutagen) derived) is still under debate, has the particularity to proliferate at high rearing temperatures, notably in the brain, thus causing the early death of the flies (Min & Benzer 1997; Rohrscheib *et al.* 2016).

→ *Because the protective effect is related to Wolbachia density, wMelPop is a strain that exacerbates both costs (physiology) and benefits (immune protection), making it easier to study the impact of stressors on this association.*

More recently, its high proliferation capacity has been linked to a genomic region containing eight genes, called Octomom (Chrostek *et al.* 2013; Chrostek & Teixeira 2015; Duarte *et al.* 2021). The Octomom region is flanked by Reverse Transcriptase repeats, which could promote the high diversity of wMelPop variants (*i.e.*, variations in the number of Octomom repeats) observed within a fly. Chrostek & Teixeira (2015) have shown that the higher the number of repeats, the higher the bacterial density<sup>5</sup>. The number of Octomom repeats is heritable and can be selected artificially (Chrostek & Teixeira 2015).

→ *Despite vertically transmitted symbionts are generally thought to be quite homogeneous in terms of genetic diversity within a host, this strain appears to exhibit genetic variation that can be used to respond to selective pressures. The measure of the mean number of octomom copies also makes it possible to follow the evolution of the bacterial composition within the host.*

In other insect symbioses:

In insect symbioses, *Wolbachia* is generally considered as a reproductive facultative parasite, but in a few cases, *Wolbachia* evolved as an obligate symbiont, raising the question of the mechanisms underlying this dependence. During my research, I have worked (or am currently working) with two different cases where *Wolbachia* became obligate.

- ***Asobara tabida*** is a parasitoid wasp that is infected by three strains of *Wolbachia*, one of them (**wAtab3**) being necessary for oogenesis completion (Dedeine *et al.* 2001). Cytological analyses have shown that wAtab3 interferes in the wasp ovaries with programmed cell death (PCD), which modulates egg production on oogenetic checkpoints (Pannebakker *et al.* 2007). Characterizing how *Wolbachia* interferes with PCD led to highlight the importance of iron modulation by *Wolbachia* (Kremer *et al.* 2009), and suggested that the study of symbionts should be generally considered on host physiology and metabolism.

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<sup>5</sup> Duarte *et al.* (2021) recently showed that the absence of this region in variants such as wMelOctoless (obtained by mutagenesis) also causes the hyperproliferation of the strain.

→ Studying the mechanisms underlying dependence through iron modulation opened the field of the role of oxidative stress regulation in insect-*Wolbachia* symbioses.

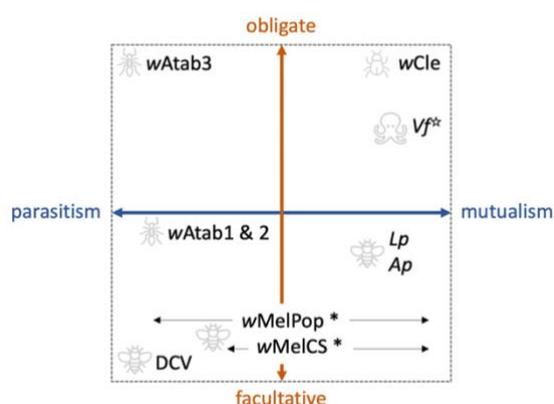
• ***Cimex lectularius*** is a bedbug that feeds exclusively on blood. As other strict hematophagous insects, *C. lectularius* is associated with an obligate symbiont that supplies its host with essential B vitamins lacking in the blood (Duron & Gottlieb 2020). Interestingly in the bedbug, this symbiont is *Wolbachia*, which in this case is a nutritional symbiont (Hosokawa *et al.* 2010), as in filarial nematodes. → Studying how *Wolbachia* evolved to become an obligate nutritional symbiont in response to diet unbalance is of great interest to determine how environment can drive rapid transitions towards mutualism and dependence.

The fruit fly as a “simplified ecosystem”

Coming back to the fruit fly, we did not restrict our studies to different *Wolbachia* strains in *Drosophila melanogaster*, but also investigated multiple interactions between *Wolbachia* and a couple of bacteria belonging to the resident gut microbiota, in a context of infection by the *Drosophila C virus* (DCV)<sup>6</sup>. Last decade, many research programs have studied the impact of specific gut bacteria on fly nutrition and gut immunity, or intestinal tissue homeostasis (reviews in Broderick & Lemaitre 2012; Grenier & Leulier 2020). Here, we mainly will focus on two bacterial species that are commonly found in fly gut (in the wild and in laboratory stocks) and very abundant in the microbiota. These bacteria act as major modulators of host metabolism at the juvenile stage. Indeed, ***Lactobacillus plantarum***<sup>7</sup> (*Lactobacillaceae*) and ***Acetobacter pomorum*** (*Acetobacteraceae*) synergistically increase larval growth, but provides low or no physiological benefits to adults (Storelli *et al.* 2011; Téfit *et al.* 2018; Consuegra *et al.* 2020).

→ *Drosophila melanogaster* is a useful model system to study host-gut microbiota interactions and their impact on host physiology and immunity (Douglas 2018). Indeed, it is possible to create gnotobiotic flies (*i.e.*, create germ-free flies and recolonize them with specific bacteria), in order to dissect fine interactions between symbiotic partners.

To summarize, **Figure 3** shows the diversity of the symbiotic systems I studied in terms of impact on the host and dependence on the symbiont, and **Table 1** presents the main characteristics of these symbiotic systems.



**Figure 3 | Position of symbiotic systems I studied along the continuum between parasitism and mutualism (blue), and according to the level of dependence of the host (orange). Host: grey drawing, symbiont: name.**

\*: variable depending on environmental conditions (*e.g.*, presence/absence of virus), ☆: ecological (but not physiological) dependence.

wAtab: *Wolbachia* from *Asobara tabida*, wCle: *Wolbachia* from *Cimex lectularius*, wMelCS and wMelPop: *Wolbachia* from *Drosophila melanogaster*, Lp: *Lactobacillus plantarum*, Ap: *Acetobacter pomorum*, Vf: *Vibrio fischeri*, DCV: *Drosophila C virus*.

<sup>6</sup> The impact of DCV on fly physiology and life-history traits will be presented in chapter 3.1, as well as the potential interference between DCV and {*Wolbachia* ± the gut commensals} (chapter 3.2).

<sup>7</sup> also reclassified as *Lactiplantibacillus plantarum*.

Host	Symbionts	Transmission mode	Type of interaction	Dependency of the host
Squid ( <i>Euprymna scolopes</i> )	<i>Vibrio fischeri</i>	Horizontal	Mutualism (M)	physiologically: no
Fruit fly ( <i>Drosophila melanogaster</i> )	<i>Wolbachia</i> (wMelCS)	Vertical	Variable (O-M)	no
	<i>Wolbachia</i> (wMelPop)	Vertical	Variable (P-M)	no
	<i>Lactobacillus plantarum</i>	Horizontal	Mutualism	depends on food quality
	<i>Acetobacter pomorum</i>	Horizontal	Mutualism	depends on food quality
	<i>Drosophila C virus</i>	Horizontal	Parasitism (P)	no
Bed bug ( <i>Cimex lectularius</i> )	<i>Wolbachia</i> (wCle)	Vertical	Mutualism	yes
Parasitoid wasp ( <i>Asobara tabida</i> )	<i>Wolbachia</i> (wAtab1,2,3)	Vertical	Parasitism	Yes (wAtab3) only

**Table 1** | Main characteristics of the model systems studied.

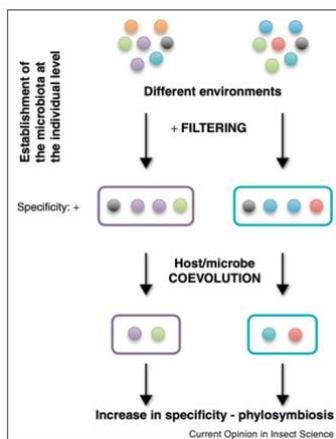


# Chapter 1 – Assembly rules

The composition of microbial communities is highly diversified and complex, and varies according to the environmental niche. Interestingly, only a fraction<sup>8</sup> of the community (*i.e.*, the microbiota<sup>8</sup>) will colonize hosts, and more precisely, specific tissues (*e.g.*, niches) within the host. This raises the question of how certain microbes acquired in the environment are filtered out and colonize the host tissues: are these those present in the host diet/near environment, or those able to digest this diet and which have been selected for? Are those that can survive (*e.g.*, pH, O<sub>2</sub>, antimicrobial cocktails, ...) in this new niche or on their way in? Are those with specific receptors or chemical mechanisms to be attracted?

More generally, to what extent does the host select its microbiota? How coevolutionary processes increase host-microbiota specificity and potentially phylosymbiosis? Which molecular/chemical mechanisms can help selecting specific microbes? How can the host-microbiota interactions shape both immunity and development and stabilize the interaction?

The answers to these questions will be different depending on the variability of the local environment (diet, temperature...), but also on the symbiotic relationship with the host. For instance, frequent changes in diet during life or during development can strongly modulate the gut microbiota composition and limit a potential adaptation to the environment. Partner choice and development of symbiotic organs are in turn mainly observed in mutualistic associations, as they participate in the stabilization of the interaction.



We wrote with Fabrice Vavre a mini-review in 2014 in *Current Opinion in Insect Science* ([Vavre & Kremer 2014](#)), exploring the ecological factors shaping microbiota composition, the process of active filtering through immunity resulting in higher host/microbiota specificity, the mechanisms involved in niche construction (*i.e.*, the development of a dedicated symbiotic organ) that favor long-term coevolution, but also the impact of the microbiota on behavioral traits that can favor isolation, diversification and speciation.

**Figure 4 | Processes of variation in microbiota composition.**

Circles of different colors represent bacterial strains/species and rectangles host genotypes ([Vavre & Kremer 2014](#)).

In this chapter, I will describe two examples of “assembly rules” of the microbiota in biological systems evolving in contrasting environment. The first one will focus on the factors influencing the establishment of the bacterial and fungal communities in the fruit fly larvae, whereas the second one will present molecular and chemical mechanisms involved in the establishment of the specificity between the bobtail squid and a bioluminescent bacterium from the bacterioplankton.

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<sup>8</sup> Many definitions have been proposed concerning microbiota and microbiome, and we will use here the definitions proposed in Berg *et al.* 2020. The **microbiota** (from Greek, *μικρος* and *βιοτα*) will refer to all small living organisms of an ecosystem or a particular area. The **microbiome** (from Greek, *μικρος* and *βιος*, and modified by the “ome” anglicization ending) will also include their “theatre of activity” (*i.e.*, structural elements, metabolites/signal molecules, and the surrounding environmental conditions).

## Related publications:

Vavre F, Kremer N. (2014) Microbial impacts on insect evolutionary diversification: from patterns to mechanisms. *Curr. Op. Insect. Sc.* 4:29-34. <https://doi.org/10.1016/j.cois.2014.08.003>

### 1.1 - Establishment of the gut symbiotic community in *Drosophila melanogaster* larvae

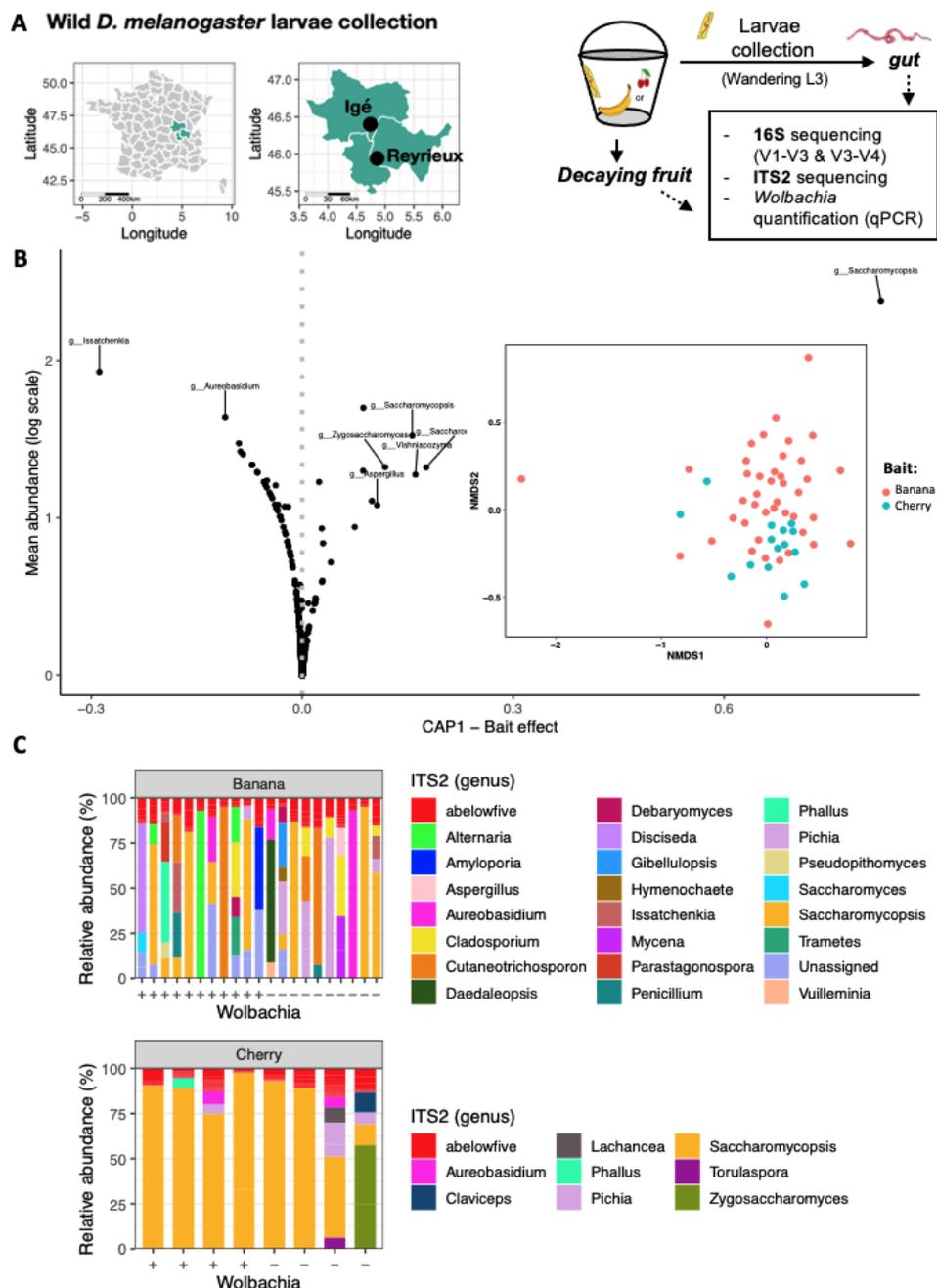
Gut microbiota plays a major role in insect physiology and ecology, such as nutrition, immunity or communication. Gut microbiota composition can be influenced by many factors (see general review in Engel & Moran 2013) such as diet, morphology and physio-chemistry of the gut compartments (pH, Oxygen availability, redox potential, immune response)... In fruit flies, no core microbiota has been evidenced, as the composition of gut microbiota appears to strongly vary within and among *Drosophila* populations and species (Wong *et al.* 2013), suggesting a large influence of the environment in shaping the gut microbiota. Recently, it has been shown that in addition to food-substrate, temperature and host population structure also correlate with microbiome structure in adult flies collected in Europe (Wang *et al.* 2020). In *D. melanogaster*, bacterial communities seem to perpetually re-associate with environmentally acquired microbes (in the lab) through ingestion of the surrounding nutritive substrate and its excretion (Téfit *et al.* 2018). The association between *D. melanogaster* and the wild isolated *Acetobacter thailandicus*, shown to be mutually beneficial, is also stable over generations through microbial farming (Pais *et al.* 2018).

Because many insects, among which *Drosophila melanogaster*, can harbor endosymbionts such as *Wolbachia*, this tackles the question of the potential impact of this bacterium on the structuration of the microbial community (Brinker *et al.* 2019). In natural populations of mycophagous drosophilids, the abundance of *Wolbachia* is co-correlated with host gene expression and with the relative abundance of certain bacterial orders, suggesting an interdependence between *Wolbachia* abundance, host functional traits and microbiota composition (Fromont *et al.* 2019). *Wolbachia* also impacts the abundance of many bacterial taxa in the terrestrial isopod *Armadillidium vulgare* (Dittmer & Bouchon 2018), in the cabbage root fly *Delia radicum* (Ourry *et al.* 2021) or in *Drosophila nigrosparsa* (Detcharoen *et al.* 2023). In lab strains of *D. melanogaster*, *Wolbachia*-infected adult flies harbor significantly reduced titers of *Acetobacter*, and the presence of *Wolbachia* appears to be an important factor shaping microbiome composition throughout fly development (Simhadri *et al.* 2017).

These studies show that the presence of *Wolbachia* can significantly impact the gut microbiota of flies. However, they only focus on adult flies, which feed significantly less than larvae and disperse more. As larvae are mostly sedentary on their fruit, the microbial composition of the fruit could have a strong impact on the microbial composition of the digestive tract of *Drosophila*. Also, because fruit flies grow on rotten fruits, it is important to study not only bacterial community but also fungal community. Within the ComEndoVir project (funded by the Labex Ecofect and developed together with François Leulier (IGFL) and Vincent Raquin (Post-Doc now assistant professor at IVPC)), we therefore sought to determine whether the composition of the gut microbiota of wild *Drosophila melanogaster* larvae differs according to *Wolbachia* infection status and/or trapping site and/or nutrient substrate.

We carried out field campaigns at two different sites in the Rhône valley (Igé, Reyrieux, **Figure 5A**), and captured *Drosophila* larvae that had developed on two different substrates (banana, cherry). Previously, during Marine Vallat's IUT internship, we set up a method for typing *Drosophila* larvae using RFLP (Restriction Fragment Length Polymorphism), which enables us to differentiate between the different species of *Drosophila* larvae (Raquin *et al.* 2018). We dissected larval guts, and the carcass was used to determine *Drosophila* species and *Wolbachia* infection status. *Wolbachia* infecting ~50%

of the larvae collected with a similar load between sites and fruits, we selected 10 and 4 larvae per site for the banana and cherry samples, respectively, to sequence individual guts for both bacterial (16S, V1-V3 and V3-V4 regions) and fungal (ITS2) communities (collaboration with H el ene Henri and the BIOfidal sequencing platform). The analysis pipeline has just been completed (with the help of H el ene Henri (LBBE) and Guillaume Minard (LEM)), and Amplicon Sequence Variants (ASV) are still under analysis. Rarefaction analyses indicate that the initial sequencing depth was sufficient (100,000 reads/sample), even for *Wolbachia*-infected individuals (sometimes representing over 90% of reads). Preliminary analyses on fungal ASV indicate that: 1) fungi develop on rotten fruits and are mostly absent from fresh fruits; 2) fungal community highly diverges between rotten fruits and fly guts; 3) both fruit type and collection site structure fungal community ( $p < 0.001$ , example on baits in **Figure 5B**), whereas *Wolbachia* does not impact fungal structure; 4) Fungal diversity is higher in banana compared to cherries (**Figure 5C**, Raquin et al. in prep #1).



**Figure 5 | Microbial gut community of wild *Drosophila melanogaster* larvae (preliminary results on fungi).**  
**A.** Experimental set-up for the collection and processing of wild *D. melanogaster* larvae.

**B.** Example of Constrained Analysis of Principal Coordinates exhibiting ASV candidates (CAPscale, left), and non-metric multidimensional scaling (NMDS, right) analyses on ITS-defined ASV, focusing on the “bait” factor. **C.** Example of abundance of ITS-defined ASV in Igé, structured by fruit type (and *Wolbachia* infection status). Figures from Guillaume Minard (B) and Vincent Raquin (A, C).

These data are currently being analyzed (notably alpha- and beta-diversity based on V1-V3 and V3-V4 sequencing, CAPscale and NMDS analyses) but suggest that the (biotic) environment plays an important role in defining larval gut microbiota. Indeed, other insects wandering over the baits may have seeded the fruits with different microbes, influencing the final larval gut community composition. Future studies could focus on how the composition of the larvae microbiota modifies the gut transcriptional function (as it has been done in Bost *et al.* 2018), and participates (or not) in local adaptation or plastic response towards the use of food resources. Pioneer studies using pulse-chase protocols and microscopy have also started to investigate the stability of different strains in the adult gut (Dodge *et al.* 2023). Similar experiments performed in the larval gut of gnotobiotic flies could help defining the initial colonization steps that stabilize gut microbiota according to the initial composition of the fruit. Finally, addressing how gut community changes after metamorphosis would help to understand the stability of such communities.

#### **Related publications:**

Raquin V, Henri H, Minard G, Leulier F, Kremer N. Influence of diet, trapping site and *Wolbachia* on bacterial and fungal communities of fruit fly larvae. *In preparation #1*.

Raquin V, Henri H, Vallat M, Leulier F, Gibert P, Kremer N. 2018. Development of a PCR-RFLP assay to identify *Drosophila melanogaster* among field-collected larvae. *Ecology and Evolution*. <https://www.doi.org/10.1002/ece3.4453>

## 1.2 - Selection mechanisms in the squid/vibrio symbiosis

The stability of a mutualistic association can be explained by the fidelity of the partners, which generally leads to an alignment of interests between them (Sachs *et al.* 2004). From the host side, different mechanisms can be selected for and lead to what is generally called “partner choice”:

(i) **Selective incorporation** occurs when only a subset of the microbes present in the environment can enter within the host. For instance, In the bean bug *Riptortus pedestris*, the intestine contains a constricted region, the ‘sorting organ’, which discriminates *Burkholderia* symbionts from non-symbiotic bacteria such as *Escherichia coli* (Ohbayashi *et al.* 2015).

(ii) **Selective accommodation** occurs when all microbes enter within the host, but only part of them can survive within this new niche. For instance, Dual oxidases (DUOX) play an important role in microbe homeostasis in *Drosophila*, through the detection of Uracil. Indeed, gut symbionts do not produce Uracil whereas pathogens do. This Uracil is a ligand of DUOX, whose activation results in the local production of Reactive Oxygen Species (ROS), the elimination of pathogenic bacteria, and the repair of damaged intestinal cells (Lee *et al.* 2013).

(iii) **Selective maintenance** occurs when there is an active process from the host side to select for the best symbionts (*i.e.*, those that give a maximal benefit), and sanction the cheaters. For instance, in mutualistic rhizosphere associations, the plant adjusts the quantity of O<sub>2</sub> required for *Rhizobium* reproduction and nodule development depending on the symbiont performance (estimated by the ability to fix N<sub>2</sub>) (Kiers *et al.* 2003, 2011).

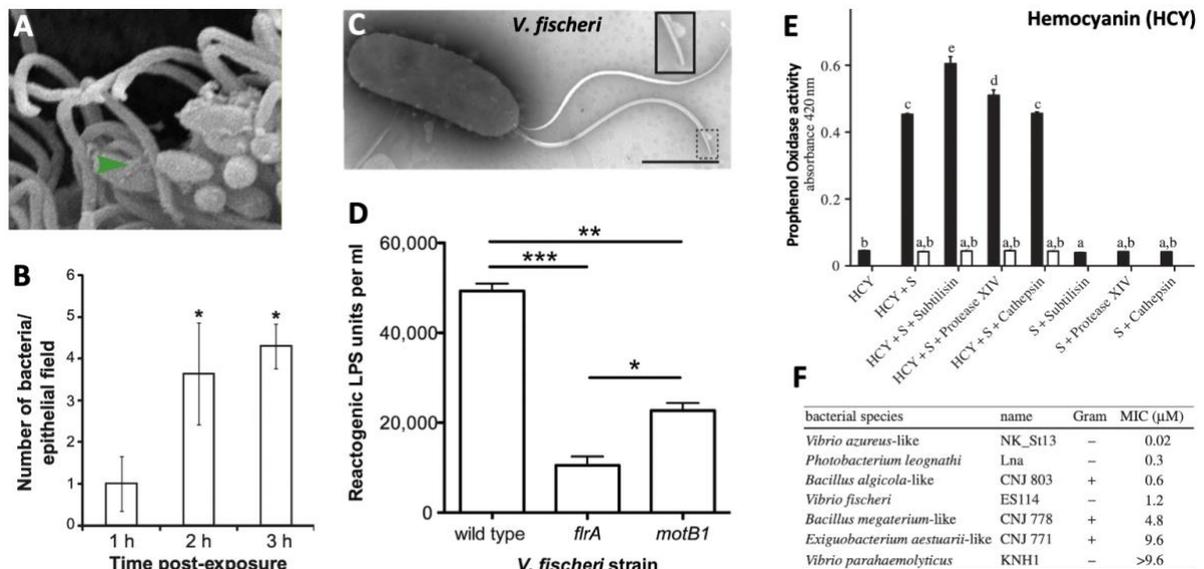
These selection processes involve that the partners can recognize each other and 'communicate' (*via* chemicals, changes in gene expression in specific signaling pathways), but also that mechanisms able to exclude (*i.e.*, *via* antimicrobial substances) or to sanction (*i.e.*, *via* metabolic restriction) microbes that will not be part of the final symbiotic association have been selected for to stabilize the association.

In this section, we will primarily focus on the selective incorporation and accommodation processes. To go deeper in the characterization of such mechanisms, simple symbiotic associations that have co-evolved, such as the squid-vibrio association, are perfect model systems. In this association between *E. scolopes* and *V. fischeri*, the fact that *V. fischeri* represents less than 0.5% of the bacterioplankton raises the questions of how *V. fischeri* is recognized, how it becomes exclusive and migrates to the light organ?

In order to understand how *V. fischeri* and only *V. fischeri* initiates symbiosis with the bobtail squid, I first participated in the physical and chemical characterization of this contact. The physical contact proceeds in two steps: the attachment of bacteria to the cilia of the epithelium, then the aggregation of bacteria in the mucus before migration through the pores of the light organ (Yip *et al.* 2006).

In Margaret McFall-Ngai's lab, we decided to use realistic environmental conditions (~ 5000 CFU of labelled *V. fischeri* / mL of unfiltered seawater containing ~10<sup>6</sup> environmental bacteria) to determine how many cells are in contact with the ciliated epithelium 3h after hatching. We showed that only 5 *V. fischeri* bacterial cells attached to the ciliated surface of the light organ, a process that required the bacteria to be alive (Altura *et al.* 2013) (Figure 6A,B). This contact induced the trafficking of hemocytes into the blood sinus of the ciliated epithelium, which suggests that bacterial components were recognized in this early step of the interaction. Caitlin Brennan from Ned Ruby's lab then explored in depth the role of the rotation of the flagellum in the release of bacterial-derived lipopolysaccharides (LPS) that could serve as signals recognized by specific receptors such as LBPs (Krasity *et al.* 2011; Chen *et al.* 2017). We showed that, indeed, the rotation of the flagellum releases (Figure 6C,D). In addition, while motility mutant were still able to colonize the light organ, they did not induce apoptosis involved in the regression of the light organ appendages, showing that LPS released by the rotation of the bacterial sheathed flagellum triggers the regression of structures involved in bacterial recruitment (Brennan *et al.* 2014), thus limiting subsequent colonizations.

Immediately after hatching, the contact of the ciliated epithelium with peptidoglycans present in the sea water induces the shedding of mucus (Nyholm *et al.* 2002). Mucus contains numerous structural molecules, such as mucins, which may facilitate the recruitment of symbionts. It also contains potentially bactericidal molecules, such as nitric oxide (Davidson *et al.* 2004), which may play a role in the exclusion of non-symbiotic bacteria. Other proteins could be involved in the creation of this anti-microbial cocktail, in which *V. fischeri* would be more resistant than other bacterioplankton bacteria. For example, I have shown that hemocyanin is highly expressed in mucus and has a phenol oxidase activity upon proteolytic activation, which is toxic to certain marine bacteria (Kremer *et al.* 2014) (Figure 6E,F).



**Figure 6 | Physical and chemical interactions of *V. fischeri* in the mucus covering the ciliated epithelium.**

**A.** Direct contact (TEM, green arrow) between cilia and bacterial cells. **B.** Between 4 and 5 cells aggregate in the epithelial field when *V. fischeri* is present at  $5 \times 10^3$  CFU/mL in environmental seawater.

**C.** Dissociation (TEM) of the sheath of a *V. fischeri* flagellum, forming a vesicle-like structure (box). **D.** Wild-type *V. fischeri* strains release more reactogenic LPS than non-motile strains.

**E.** Hemocyanin (HCY) exhibits prophenol-oxidase (PO) activity in presence of the dopamine substrate (S) and various proteases (black bars). This PO activity is inhibited in presence of PO-Inhibitor (white bars). **F.** HCY exhibits antimicrobial activity (expressed as Minimal Inhibitory Concentrations) in the presence of Dopamine.

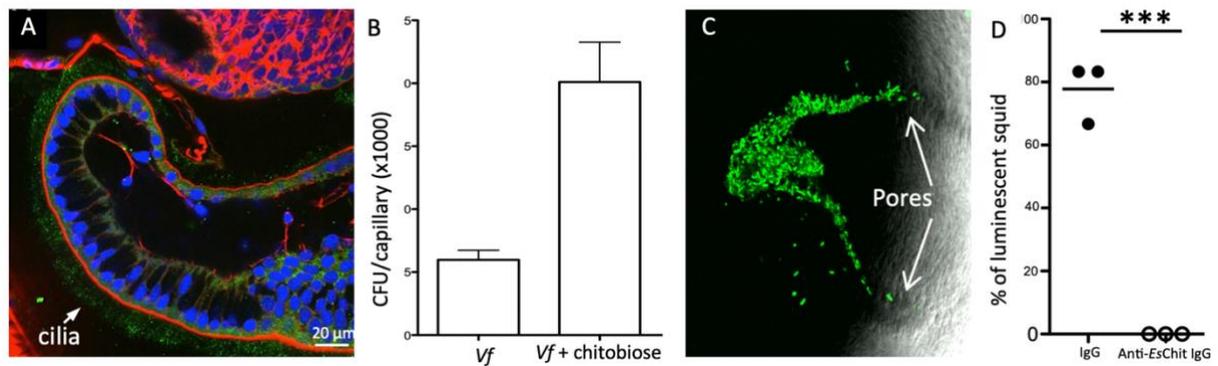
A, B: from [Altura et al., 2013](#); C, D: from [Brennan et al., 2014](#); E, F: from [Kremer et al., 2014](#).

In a second step, I characterized the molecular events that take place during the very first contact between partners at the level of the muco-ciliated epithelium of the luminal organ. As we showed that only 5 bacteria aggregate on the epithelium of the light organ, we wanted to further determine whether these few bacteria modify host gene expression. I thus initiated a comparative transcriptomics approach, in collaboration with Philip Rosenstiel (University of Kiel, Germany). High-throughput sequencing (454) was performed between symbiotic and aposymbiotic individuals 3 h after emergence ( $\pm V. fischeri$ ). After *de novo* assembly, 21,966 contigs were defined (mean length  $\sim 700$  bp; 47.3% annotated) and constituted the first light organ reference transcriptome at 3 h. Using differential analysis between symbiotic and aposymbiotic light organs, we showed that the host modifies the expression of its genes after contact with the symbiont, particularly those related to sugar and peptide degradation activities ([Kremer et al. 2013](#)).

Among these differentially expressed genes, I chose to focus **chitotriosidase** (*i.e.*, the enzyme that degrades chitin to chitobiose), since the presence of a chitobiose-attracting chemoattractant gradient was known to be necessary for *V. fischeri* migration through the pores of the luminal organ ([Mandel et al. 2012](#)), **Figure 7C**). Using biochemical approaches, I showed that the protein is present in mucus and at the pore level (**Figure 7A**), and that it is primarily active in the pH range present in mucus. In collaboration with Caitlin Brennan, we also showed that *V. fischeri* is not attracted *in vitro* to a chitobiose gradient if it has not been “primed”<sup>9</sup> with chitobiose (**Figure 7B**), suggesting that it must be ‘sensitized’ by host chitobiose in the light organ. In this context, squid colonization efficiency appeared to be drastically reduced when I inhibited chitotriosidase using specific antibodies (**Figure 7D**). All

<sup>9</sup> Be in direct contact with the molecule

together, these results suggest that the over-expression of chitotriosidase is induced by the first contact between partners and participates in: (i) creating the chitobiose gradient and (ii) making *V. fischeri* sensitive to this gradient, thus facilitating symbiont migration through the pores of the luminous organ. This study was pioneering in terms of the analysis of molecular dialogue during the initiation of a symbiotic association. The results showed that initial contact with a small number of symbionts enables host genes to be reprogrammed, promoting the establishment of specificity.

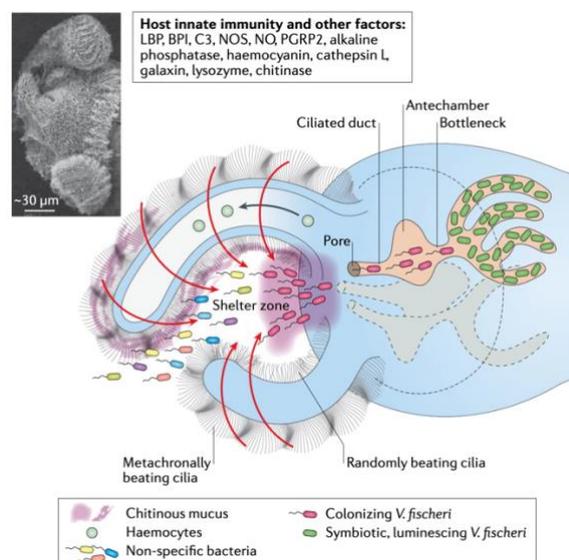


**Figure 7 | Influence of chitotriosidase in establishing specificity after the initial contact between *E. scolopes* and *V. fischeri*.**

**A.** Localization of chitotriosidase, mainly at the level of the ciliated epithelium (chitotriosidase: green; nucleus: blue; cytoskeleton: red). **B.** *in vitro* chemoattraction of *V. fischeri* towards chitobiose. Bacteria were pre-cultured in chitobiose-free and chitobiose-containing medium. **C.** Migration of *V. fischeri* (green) through the pores of the light organ. **D.** Inhibition of colonization after reduction of chitotriosidase activity (anti-EsChit IgG condition). From [Kremer et al., 2013](#).

Among the candidate genes differentially regulated after the contact with *V. fischeri*, we also detected host genes that could play an important role in the establishment of the symbiosis. In particular, we characterized a cysteine protease called **cathepsin L**, its localization and apoptotic-inducing activity during the first 96h following colonization. We showed that this protein plays a critical role in the symbiont-induced cell death initiating the regression of the light organ appendages early during the onset of symbiosis ([Peyer et al. 2018](#)).

To conclude, the work presented above, together with many studies performed in Margaret McFall-Ngai and Ned ruby's labs, enable to draw a model of the initial colonization process, based on a combination of biomechanical and biochemical mechanisms (**Figure 8**, Nyholm & McFall-Ngai 2021). The mucus facilitates the adhesion of bacteria on the ciliated epithelium and the beating of the ciliated epithelium favors a current that moves bacteria in a shelter zone, at the vicinity of the pores. The contact with few bacterial cells and the release of MAMPs favors the creation of a chemo-attractant gradient and up-regulates proteins with antimicrobial activities. Together, they form a microenvironment that incorporates selectively *V. fischeri* and prevents other non-symbiotic bacteria to colonize. This colonization process also induces



**Figure 8 | The winnowing of *Vibrio fischeri* in the light organ of the squid *Euprymna scolopes*.**  
From Nyholm & McFall-Ngai 2021.

morphological changes, such as the regression of the ciliated epithelium through apoptosis, which also limits subsequent colonizations. After migration through the pores to the antechamber and the crypts, *V. fischeri* grows to reach a density that enables light production.

### **Related publications:**

**Kremer N**, Philipp EER, Carpentier M, Brennan CA, Kraemer L, Altura MA, Häsler R, Heath-heckman EAC, Peyer SM, Schwartzman J, Rader BA, Ruby EG, Rosenstiel P, Mcfall-Ngai MJ (2013) Initial Symbiont Contact Orchestrates Host-Organ-wide Transcriptional Changes that Prime Tissue Colonization. *Cell Host Microbe*, 14, 183–194. <https://doi.org/10.1016/j.chom.2013.07.006>

Preview:

Wernegreen J (2013) First Impressions in a Glowing Host-Microbe Partnership. *Cell Host Microbe*, 14, 121–123. <http://dx.doi.org/10.1016/j.chom.2013.07.015>

**Kremer N**, Schwartzman J, Augustin R, **Zhou L**, Ruby EG, Hourdez S, Mcfall-ngai MJ (2014) The dual nature of haemocyanin in the establishment and persistence of the squid – vibrio symbiosis. *Proc. R Soc. Lond. B*, 281, 20140504. <https://doi.org/http://dx.doi.org/10.1098/rspb.2014.0504>

Altura MA, Heath-Heckman EAC, Gillette A, **Kremer N**, Krachler A-M, Brennan C, Ruby EG, Orth K, McFall-Ngai MJ (2013) The first engagement of partners in the *Euprymna scolopes-Vibrio fischeri* symbiosis is a two-step process initiated by a few environmental symbiont cells. *Environ Microbiol*, 15, 2937–50. <https://doi.org/10.1111/1462-2920.12179>

Brennan CA, Hunt JR, **Kremer N**, Krasity BC, Apicella MA, McFall-Ngai MJ, Ruby EG (2014) A model symbiosis reveals a role for sheathed-flagellum rotation in the release of immunogenic lipopolysaccharide. *eLife*, 3, e01579. <https://doi.org/10.7554/eLife.01579>

Peyer S, **Kremer N**, McFall-Ngai M. 2018 Involvement of a host Cathepsin L in symbiont-induced cell death. *MicrobiologyOpen*. e632. <https://doi.org/10.1002/mbo3.632>

### 1.3 - Discussion

The studies I have presented contribute to a better understanding of the establishment of communities within a host (*e.g.*, structuring genetic or environmental factors, selection mechanisms). In complex communities such as in *Drosophila*, determining pioneer or keystone species could help understanding the variability observed between individuals, sampling sites or baits.

Concerning the diversity within/between communities, we however face a limited resolution at the level of taxonomic and metagenomic diversity, especially for the fungal diversity. In terms of assembly rules, the lack of consideration for flows, spatial and temporal dynamics currently limit their study. In addition, microbe-microbe interactions (*i.e.*, competition/facilitation/exclusion) within a host or an organ should be more considered. These obstacles could be overcome by: 1) developing fungal databases with high quality assignments; 2) integrating community ecology tools (Tipton *et al.* 2019); 3) developing networks, multi-scale models or game theory; 4) artificially manipulating the biological system through the sequential reintroduction of specific species or dilutions of complex communities in axenic or gnotobiotic models.

Concerning the description of selection processes, working on binary associations greatly improves the resolution of the morphological and molecular interactions. Innovative technologies such as spatial and single-cell transcriptomics (through laser-capture microdissection) are promising techniques for better resolving interactions at the contact site. Interactions remain dynamic processes accompanied by very rapid changes and studying signal exchanges can be challenging. The use of *in vivo* imaging systems or repeated measurements can help approaching the dynamic aspect of the initial interaction/selection process.

Finally, better understanding how microbial communities are stable within individuals, and how they are transmitted to other individuals (or from one generation to the next) will help to determine the assembly rules of symbiotic associations in specific environments.



## Chapter 2 – Control & Maintenance

Once the colonization has been established within a host, the **regulation of symbiotic density** is a key factor for the stability of the symbiosis. From the host point of view, there is a trade-off between the cost associated to the presence of the symbiont (*e.g.*, use of the host resources (nutrients, cellular machinery, ...), induction of immune defenses) and the potential benefits associated to their presence (*e.g.*, provision of nutrients, protection against pathogens, light production, ...). From the symbiont point of view, both virulence and transmission rely on the regulation of the symbiotic density, and lead to another trade-off (Anderson & May 1982). Indeed, an increased virulence due to high density will favor the instantaneous transmission rate of the symbiont but will shorten the host lifespan and thus the window of transmission of the symbiont. From an evolutionary point of view, when there is heterogeneity within the symbiotic population, two types of selection can thus occur (Alizon *et al.* 2013): 1) a *within host* selection, where the most competitive strain will win even if there is a strong cost to the host, which can lead to the premature host death (this selection will favor the strains that replicate the fastest); 2) a *between host* selection, where the best transmitted strain will win (this selection will generally favor strains whose density allows transmission while limiting fitness costs for the host). Because of the global alignment of interests between both partners in vertically transmitted symbioses and in mutualistic associations, an optimum density is generally selected for to optimize both offspring production and colonization by symbionts.

From the host side, various mechanisms of symbiont control have been described (Ratzka *et al.* 2012; Douglas 2014; López-Madrugal & Duarte 2019). A **fine-tuning of the immune response** can be mounted, involving notably two key immune effectors: anti-microbial peptides (AMPs) and reactive oxygen species (ROS). The presence of dedicated organs harboring symbionts favors a specific immune regulation, separated from the general immune response (Ferrarini *et al.* 2022). For instance in the cereal weevil *Sitophilus orizae* or in the tse-tse fly *Glossinia sp.*, specific regulations of the symbionts have been described in bacteriomes and milk glands, the organs harboring the nutritional symbionts (Zaidman-Rémy *et al.* 2018). This **tolerance** of the symbiont depends on the balance between costs and benefits, and varies along the development of the host in response to need for amino acids or vitamins provided by the symbionts (Rio *et al.* 2006; Vigneron *et al.* 2014). A specific regulation can also take place in the organ that transmits symbionts, notably in the ovaries in case of vertical transmission (Ferrarini *et al.* 2023). In horizontally transmitted symbioses where the nutritional symbiont requires host resources to survive, hosts can limit the proliferation of “cheaters” (*i.e.*, strains that do not provide the nutritional benefit to the host) by decreasing the **metabolic delivery** to symbionts. This is the case in plant nodules, where rhizobia that do not fix Nitrogen are sanctioned by soybeans through a reduction in O<sub>2</sub> supply, and thus decrease in density within nodules (Kiers *et al.* 2003). From the symbiotic side, density can be regulated through mechanisms that control the intrinsic proliferation rate, but also indirectly, through mechanisms that control the access to nutrient (*e.g.*, presence/absence of proteolytic enzymes that give access to a nutrient) or the tropism within tissues (Nyholm & McFall-Ngai 2004; López-Madrugal & Duarte 2019).

Although the mechanisms involved in bacterial regulation are increasingly deciphered, some remain poorly understood or unexplored. For example: 1) What is the target of bacterial regulation by the host? 2) What is the relative part of selection and drift in vertically transmitted symbioses, in which strong bottlenecks occur during transmission? 3) What is the impact of bacterial proliferation in host tissues, and what mechanisms can the host activate to limit cellular/physiological damages due to the

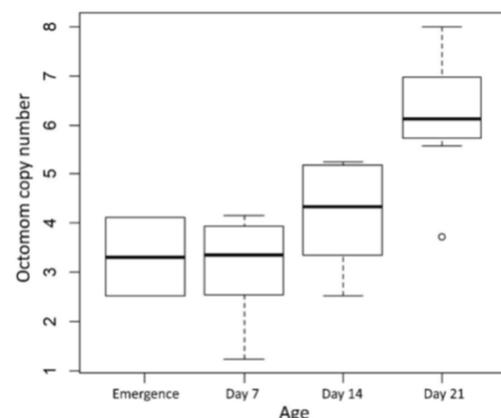
presence of symbionts, while maintaining the benefit of their presence? 4) How important are control mechanisms independent of classical immune pathways (*e.g.*, differential metabolite allocation) in the control and maintenance of symbioses? 5) To what extent does the presence of symbionts impact host physiology and the proper maintenance of symbiosis?

In this chapter, I will try to answer some of these questions, and describe two examples of control (or not) and maintenance of symbionts within their host. The first one will focus on the “control” of *Wolbachia* density in its host *Drosophila melanogaster*, and the relative influence of the host and bacterial determinism in this regulation. Instead of a clear genetic determinism of this regulation, we rather show that bottlenecks during transmission play a disproportionate effect on a master regulator of density in this symbiotic association. The second example will present molecular and chemical mechanisms involved in the maintenance of the symbiosis between the bobtail squid and its associated bacterium, and the importance of light production in this maintenance.

## 2.1 - “Control” of *Wolbachia* density in *Drosophila melanogaster*

To address this question of control, I chose to study the vertically transmitted symbiosis between *Drosophila* and the virulent wMelPop strain of *Wolbachia*, in which bacterial density plays a major role in the expression of the extended phenotype. Indeed, the greater the density, the greater the virulence associated with infection. The *Wolbachia* strain wMelPop is also particularly interesting to study, as it features a genomic region composed of eight genes (Octomom region of around 21 kB), which can be duplicated several times. Although the functions of the genes in this region are not well characterized, a correlation between Octomom copy number and *Wolbachia* density has been observed in this strain (Chrostek & Teixeira 2015). Since density partly influences virulence, the greater the Octomom copy number, the greater the virulence.

Contrary to the conventional view that populations of vertically transmitted symbionts are homogeneous within their host, we highlighted during Caroline Michaud's internship (M2 Université de Poitiers) and David Monnin's PhD (co-supervision with Fabrice Vavre and Emmanuel Desouhant) that the wMelPop population is heterogeneous within a host in terms of the number of copies of the Octomom region, a heterogeneity that is tissue-specific. This heterogeneity could induce conflict between partners, increasing within host competition between symbiotic genotypes in a process analogous to the tragedy of the commons. This heterogeneity evolved over the lifetime of individuals at high temperature (**Figure 9**), suggesting selection at the within host level (Monnin *et al.* 2021). However, certain variations in density, notably linked to temperature, appeared to be independent of Octomom, questioning the systematical reciprocal nature of the relationship between density and Octomom copy number.

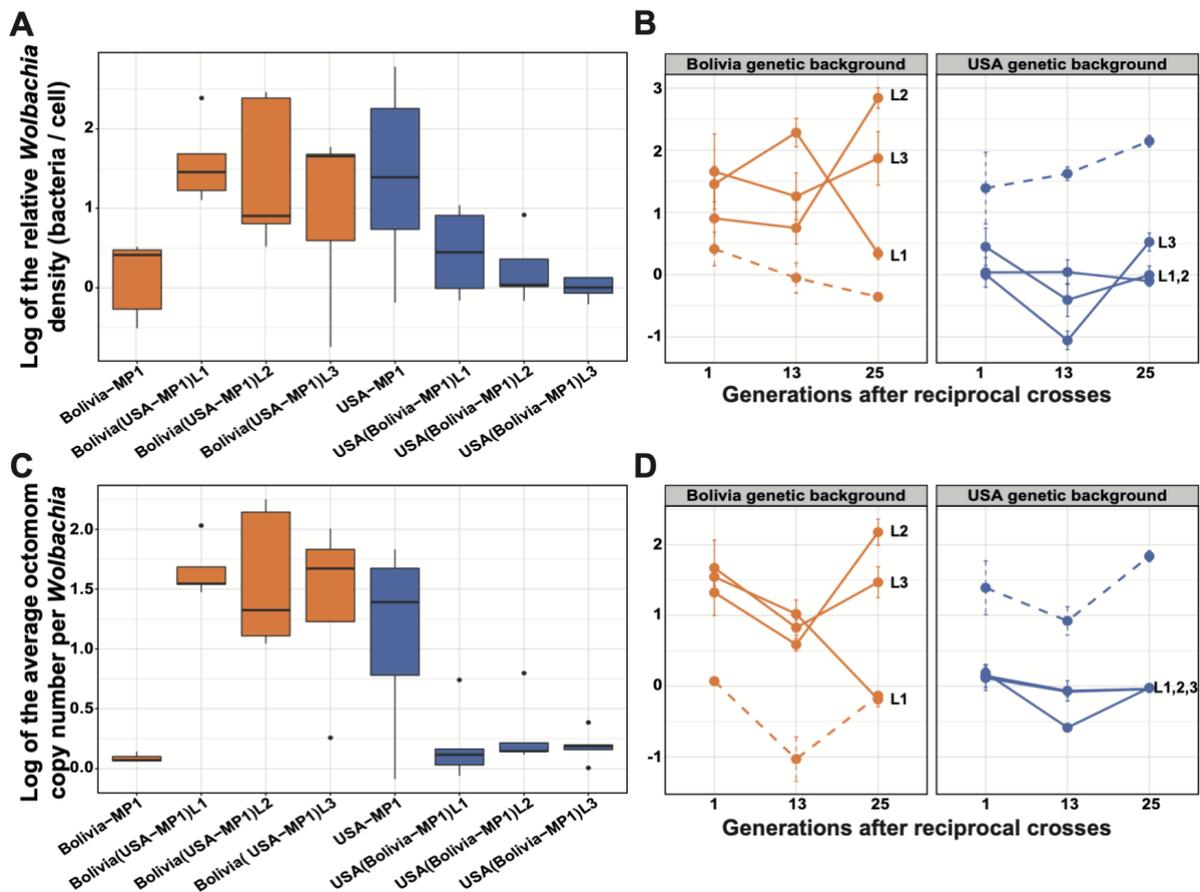


**Figure 9 | Evolution of Octomom heterogeneity with *Drosophila* age at 29°C.** *Wolbachia*: wMelPop strain. *D. melanogaster*: w1118 strain. From Monnin *et al.*, 2021.

During the master project of Alexis Bénard, we then wanted to understand the determinism of *Wolbachia* density regulation in this system, and therefore studied the influence of host and bacterial genotypes in this regulation, using populations from different localities around the world introgressed

with *wMelPop* (introgression 'MP1' with a *wMelPop* strain exhibiting a significant variability in terms of Octomom copy number, one replicate per population). We first confirmed that the Octomom copy number correlated well with the *Wolbachia* density, regardless of host genetic background - a debate currently taking place (Chrostek & Teixeira 2015, 2017 vs. Rohrscheib *et al.* 2016).

We also observed a large variability in density and Octomom copy number between the different introgressed populations, suggesting a genetic host determinism of the density, potentially through a control of the Octomom copy number. For example, the USA population had a high copy number while the Bolivia population had a low copy number. To confirm or refute an influence of the genetic host determinism on this regulation, we carried out new introgressions (several replicates per population) and performed reciprocal crosses between the two extreme populations USA and Bolivia (Bénard *et al.* 2021). In this study, we showed that: 1) in the short term, the host genotype has little influence on bacterial density, whereas the composition of the maternal bacterial gene pool has a strong influence on density in the offspring (Figure 10A,C) ; 2) in the long term, we detected a lack of directionality in the infection patterns, associated with high instability of these patterns between generations (Figure 10B,D).



**Figure 10 | Evolution of infection patterns after reciprocal crosses between lines with the most extreme density levels.**

Log of the relative density (A) or of the average Octomom copy number per *Wolbachia* (C) one generation post introgression. Evolution of the log of the relative density (B) or of the average Octomom copy number per *Wolbachia* over generations (D) (median  $\pm$  SE).  $n = 5$  flies / line / timepoint. Each color represents a host genetic background (orange: Bolivia, blue: USA) and the information in brackets represents the bacterial genetic background. Plain lines represent the replicate lineages from the reciprocal crosses (L1, L2 and L3 indicating the replicates). Dashed lines represent the lineages from the initial introgression procedure (MP1).

From Bénard *et al.*, 2021.

Given that bacterial density is strongly correlated with Octomom copy number, and that transmission of the symbiont undergoes a major bottleneck in the egg, these results suggest a strong influence of drift in this experimental system. Indeed, drift is expected to induce instability over generations through a combination of quantitative (*i.e.*, transmission of a non-equivalent number of bacteria to eggs) and qualitative/genetic (*i.e.*, random transmission of different variants) bottlenecks (Mira & Moran 2002; Galbreath *et al.* 2009; Kaltenpoth *et al.* 2010), and to lead to a random increase in the frequency of certain bacterial variants. This article therefore highlights the importance of drift in vertically transmitted symbioses, and invites us to consider drift as an important force that can limit the efficiency of within host and between host selections in natural populations, as well as the impact of bottlenecks on the host extended phenotype and on the potential of the symbiotic association to adapt to new environments.

### **Related publications:**

Bénard A, Henri H, Noûs C, Vavre F, Kremer N (2021). *Wolbachia* load variation in *Drosophila* is more likely caused by drift than by host genetic factors. *Peer Community Journal, section Evolutionary Biology* | <https://dx.doi.org/10.24072/pcjournal.50>

Recommendation:

Duncan A and Hochberg M (2021) Drift rather than host or parasite control can explain within-host *Wolbachia* growth. *Peer Community in Evolutionary Biology* | <https://doi.org/10.24072/pci.evolbiol.100126>

Monnin D, Kremer N, Michaud C, Villa M, Henri H, Desouhant E, Vavre F (2021). Experimental evolution of virulence and associated traits in a *Drosophila melanogaster* – *Wolbachia* symbiosis. *Peer Community Journal, section Evolutionary Biology* | <https://doi.org/10.24072/pcjournal.9>.

Recommendation:

Decaestecker E (2020) Temperature effects on virulence evolution of *wMelPop Wolbachia* in *Drosophila melanogaster*. *Peer Community in Evolutionary Biology*, 100111 | <https://doi.org/10.24072/pci.evolbiol.100111>.

## 2.2 - Maintenance of *Vibrio fischeri* in *Euprymna scolopes*

### 2.2.1 - Impact of symbionts on host morphogenesis and physiology

In horizontally transmitted symbioses, studies of bacterial mutants that fail to be maintained within the host help to understand the molecular mechanisms that allow bacterial symbionts to persist in host tissues, and the cellular mechanisms involved in the morphogenesis of the symbiotic organ (**Figure 11A**). In the squid-vibrio symbiosis, the mutants of particular interest are those linked to light production, an extended phenotype that plays a major role in the ecology of the squid. Light is produced by proteins encoded by the *Lux* operon, which contains 6 structural genes among which a luciferase.

Previous studies have shown that mutants of the *Lux* operon are persistence mutants: they colonize squid similarly as wild-type (WT) *V. fischeri*, but their population in the light organ is already decreased to one fourth at 48 h (Visick *et al.* 2000). The remaining question was thus to determine what happens after 48 h of interaction? Eric Koch, a PhD student in Margaret McFall-Ngai's lab, developed a protocol for rearing squid until they reach maturity (*i.e.*, around 4 weeks), which coincides with the onset of day/night activity cycles. He confirmed that the density of  $\Delta luxCDABEG$  mutants progressively declines in the light organ, whether alone or in competition with the wild-type strain (Koch *et al.* 2013). He then designed an elegant experiment in which he eliminated *V. fischeri* at specific timepoints and inoculated

them with WT bacteria two days after. He showed that, contrary to squid colonized with WT strains that cannot be recolonized after 5 days of symbiosis,  $\Delta lux$  mutants can be colonized by WT, even after 10 days of 'symbiosis'<sup>10</sup> (Koch *et al.* 2013). These results raise the question of the influence of bacterial presence on host gene regulation and cellular mechanisms, and their role in maintaining a functional symbiosis.

With the help of Eric Koch for the rearing, we thus carried out an experiment aiming to characterize changes in response to the presence of the symbiont and/or to a symbiotic event, in an established mature symbiosis (Kremer *et al.* 2018). Indeed, at 4 weeks post colonization with  $\Delta lux$ , the density of this strain was not detectable anymore by plating, suggesting that an initial colonization event occurred but the symbiosis was not maintained.

- Microscopic analysis of the luminous organ showed that morphogenesis of the outer, ciliated part was strongly accelerated by *V. fischeri* colonization, but that its adult form was largely independent of the colonization status. In contrast, changes in the shape of the inner cells (crypts) required persistent interactions between host and symbionts. Indeed, Apo / Lux epithelial cells were columnar with reduced cytoplasm, whereas WT epithelial cells were cuboidal with developed cytoplasm (Figure 11B).

- I confirmed the microscopy results by a comparative transcriptomic experiment: at 4 weeks, persistent interactions between host cells and symbionts (and not an initial colonization event) impacted on the overall host gene expression pattern compared to that observed in aposymbiotic squid light organs (Figure 11C).

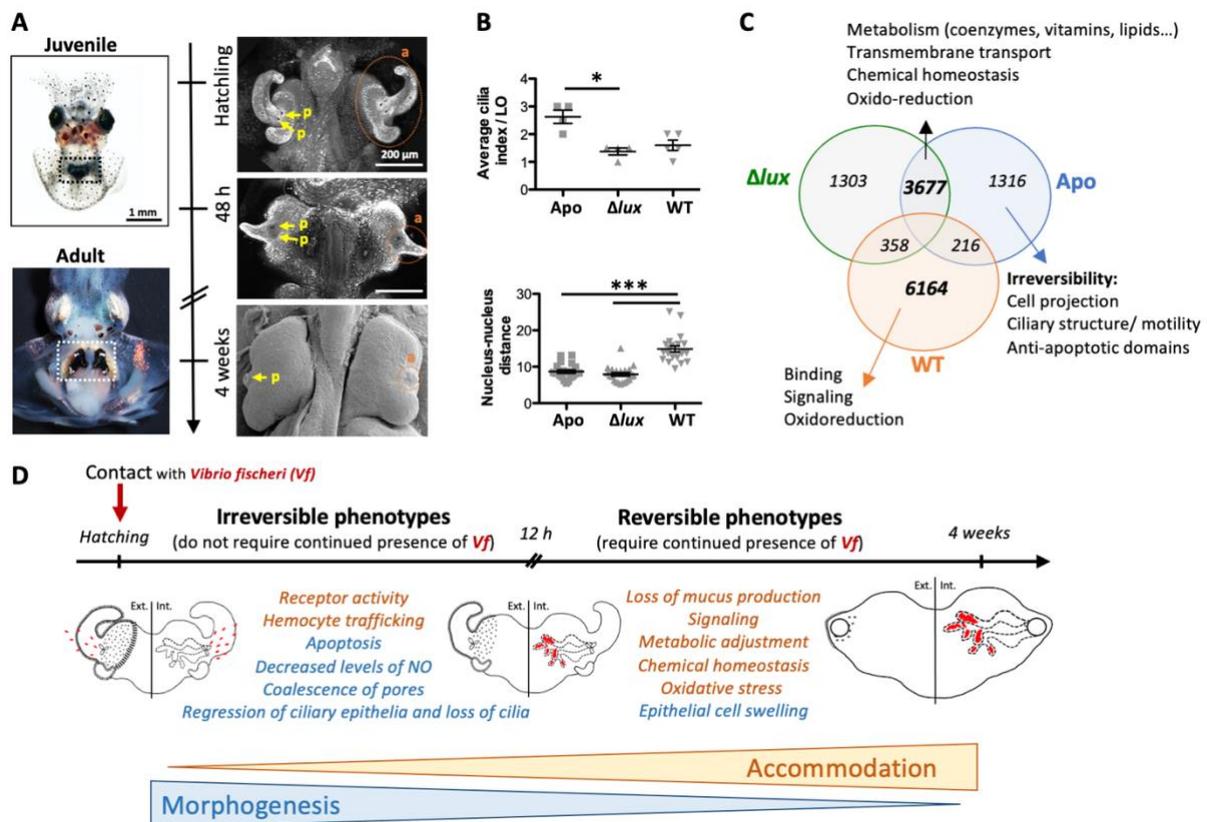
- Cilia- and microtubule-related processes were specifically over-represented in aposymbiotic squid light organs, consistent with the very slow appendage regression observed in this condition. Interestingly, membrane processes as well as numerous genes involved in the regulation of osmolites (ion transporters) and vasoconstriction were over-represented in Apo / Lux compared to WT squid light organs (details in Kremer *et al.* 2018). The absence of symbionts thus led to a disruption of cellular homeostasis, which seems to limit the 'normal' development induced by symbiosis within the light organ. On the contrary, the presence of symbionts in the light organ rather led to the repression of host activities detrimental to the production of a favorable immunological and metabolic environment.

- Together with a rotation student, Lawrence Zhou, we then characterized the transcriptomic signature of specific pathways (*e.g.*, regulation of osmolarity and oxidative stress) over the day-night cycle. Interestingly, gene expression patterns reflected the rate of symbiont release/growth throughout the day in the crypt epithelium (details in Kremer *et al.* 2018), suggesting that these regulation pathways are dynamic processes strongly depending on the presence of symbionts.

This article constitutes the first experimental study of maturation, where we showed that an early and transient exposure to the symbiont, at a time when significant tissue morphogenesis is occurring, does not leave a persistent imprint on gene expression in the mature organ. Instead, the presence of symbionts induces reversible phenotypes (Figure 11D). In particular, symbionts seem to disturb the osmotic balance of host cells through the metabolites they draw (or produce), and compensatory mechanisms to limit these disturbances (*i.e.*, accommodation) may have been selected during the long coevolutionary history between the two partners. Together, these studies can help to better define the principles underlying the host developmental dependence on its microbiota.

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<sup>10</sup> After 10 days, the density of  $\Delta lux$  is 100 times lower than that of WT strain ( $10^3$  vs.  $10^5$ , respectively)



**Figure 11 | Influence of symbionts on light organ morphogenesis and associated gene expression pattern.**

**A.** Regression of ciliated appendages of the light organ over development to adulthood. p: pores, a: appendages. **B.** Statistics on surface structures (top: cilium index, which considers the area covered by cilia and the density of long cilia, averaged per light organ) and on the light organ crypts (bottom: distance between nuclei in epithelial cells surrounding the crypt lumen). Mean  $\pm$  SE, n = 3. **C.** Venn diagram of isogroups that were over-represented after a generalized linear model analysis testing the impact of the colonization status, and the associated functions. **D.** Model of squid-vibrio interactions. *V. fischeri* (red) initially induces an irreversible morphogenetic signal that shapes the light organ. The presence of the symbiont then actively influences host genes, whose expression changes to accommodate symbionts and their long-term maintenance within the light organ. Ext., external; Int, internal. Adapted from [Kremer et al. 2018](#).

### 2.2.2 - An exchange of 'currency' between host and symbionts: rhythmicity & functionality

Light production through luminescence is the basis of the squid-vibrio symbiosis. The luciferase enzyme catalyzes the reaction  $\text{FMNH}_2 + \text{O}_2 + \text{RCHO} \rightarrow \text{RCOOH} + \text{FMN} + \text{H}_2\text{O} + \text{Light}$ <sup>11</sup>, where the bioluminescent photon production is conditioned by the intake in Oxygen. Because  $\Delta lux$  mutants are not able to persist in the light organ, I thus wondered whether the exchange between Oxygen and light is a 'currency' exchange contributing to the stability of mutualism, and how the chemical exchange between host and symbionts promotes bioluminescence.

For that purpose, I first characterized the Oxygen-carrier protein, called Hemocyanin (HCY), a huge multimeric protein over 10 kDa, each subunit containing a copper prosthetic group. Two isoforms of this protein are mainly synthesized in the branchial gland of the gills and transported in the hemolymph, then from the blood sinus in the crypts ([Kremer et al. 2014](#)). To determine the affinity of HCY for Oxygen during the day/night cycle, I established a collaboration with Stéphane Hourdez at the

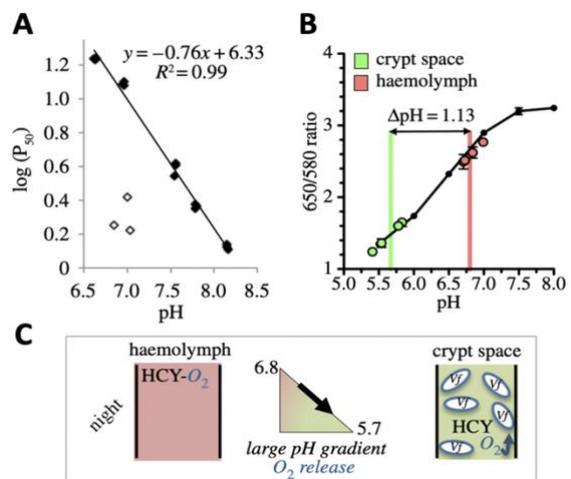
<sup>11</sup> FMNH<sub>2</sub>: reduced flavin mononucleotide; RCHO: Long-chain aliphatic aldehyde; FMN: oxidized flavin; RCOOH: carboxylic acid

*Station Biologique de Roscoff* (France). We showed that (**Figure 12**): 1) affinity of HCY for Oxygen decreases when pH drops (*i.e.*, Bohr effect), 2) pH is lower in crypt space than in hemolymph, 3) HCY affinity is two to three orders of magnitude lower than that of *V. fischeri* luciferase and cytochromes. These results suggest that, during the night, HCY helps delivering Oxygen from the blood to the symbionts.

The next question was thus to determine if acidification could drive the bioluminescent rhythm. Previous studies had shown that *V. fischeri* respire glycerol during the day and ferments chitin during the night (Wier *et al.* 2010). Julia Schwartzman pursued this work (Schwartzman *et al.* 2015) and showed that hemocytes are the main source of chitin, they migrate daily into the light organ before nightfall, where a chitinase degrades chitin in acidic by-products (chitin oligosaccharides, COS). Because an acid tolerance response was only induced at night, it suggests that the nocturnal acidification of the crypts is due to symbiont chitin metabolism.

All together, these results highlight a complex crosstalk between host and symbionts, involving many players (immune cells, carbon sources, pH, Oxygen and light) and a regulation varying over the day-night cycle. Mutualism in such horizontally transmitted symbioses thus requires many coevolutionary cross-talks, and questions their role in the stability of the symbiosis. Is the non-persistence of 'cheaters' that do not produce light (such as  $\Delta lux$ ) a result of a sanction from the host side or just a by-product of the accumulation of acids and Oxygen that become toxic in absence of functional luciferase?

In the mature symbiosis (see **Figure 11C**), among genes that were differentially over expressed between WT and Apo/ $\Delta lux$ , we also found numerous genes associated with redox processes and some genes of particular interest in the squid-vibrio symbiotic association. For example, the alkaline phosphatase, which I helped characterize previously (Rader *et al.* 2012), is an enzyme that detoxifies bacterial determinants (LPS). By dephosphorylating LPS, it could limit host immune and apoptotic responses associated with high symbiotic density overnight, and have a preventive inflammation-limiting activity, which would promote symbiont maintenance and limit its deleterious effects. These data should be considered in tandem with those obtained on the Peptidoglycan-Recognition Protein 2 (PGRP2), known to inactivate bacterial peptidoglycans on a daily rhythm in the crypts (Troll *et al.* 2010). Interestingly, both enzymes are pH-dependent, and the acidification of the crypt space described before venting (because of the catabolism of chitin) would make them less active in detoxifying LPS and PGN, making bacteria more 'pathogenic'. This would fit with the inflammatory pattern observed in crypts cells around venting. Together, these two enzymes thus participate in the 'taming' of microbe-associated molecular patterns (MAMPs) exposed by *V. fischeri*, and the tolerance of the



**Figure 12 | Characteristics of hemocyanin (HCY) and its role in the delivery of Oxygen to the symbionts.**

**A.** Determination of the Bohr effect. Oxygen affinity of HCY was measured after exchange of the blood with stabilization buffers (closed diamonds) and in native blood samples (open diamonds).

**B.** Determination of pH in circulating blood (red) and in the crypt space (green) using the pH-sensitive dye SNARF. Mean  $\pm$  SE, n = 5.

**C.** Model for the directional transport of Oxygen into the crypt space during the night: a decrease in pH in the crypt space should favor the offload of Oxygen from HCY and its use by the bacterial luciferase.

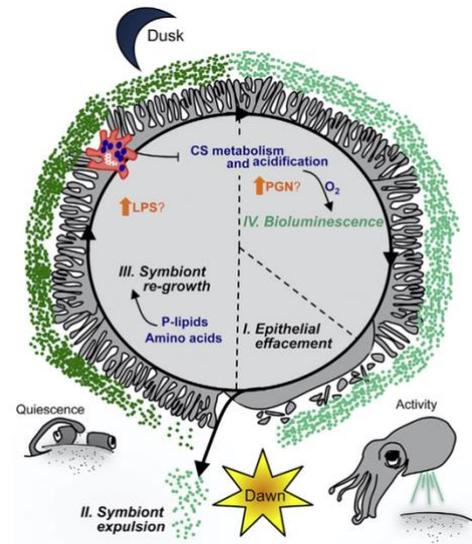
From Kremer *et al.* 2014.

bacterium in the crypt space before dawn, when the squid is active for hunting and when bioluminescence could be beneficial for its behaviors (Figure 13).

**Figure 13 | Model describing biological rhythms involved in the mature squid-vibrio symbiosis.**

Host-derived nutrients, including amino acids and phospholipids (P-lipids) support bacterial proliferation during the day. Just before dusk, several events take place: lipopolysaccharide (LPS) levels in the crypt increase due to a decrease in LPS-degrading, host alkaline phosphatase production; in addition, chitin sugar (CS)-bearing hemocytes migrate into symbiont-colonized light organ tissues. (IV) At dusk, the symbiont population ferments CS, producing acid, and releasing oxygen from the host carrier-protein, hemocyanin. The oxygen released from hemocyanin fuels bioluminescence, while the acidification of the crypts may lead to an increase in PGN levels due to its ability to decrease PGRP2 activity.

Figure and legend from Schwartzman & Ruby 2015.



### Related publications:

**Kremer N**, Koch EJ, El Filali A, **Zhou L**, Heath-Heckman E.A.C, Ruby E.G, McFall-Ngai M.J (2018) Persistent Interactions with Bacterial Symbionts Direct Mature-Host Cell Morphology and Gene Expression in the Squid-Vibrio Symbiosis. *mSystems*. 3:e00165-18 | <https://doi.org/10.1128/msystems.00165-18>

**Kremer N**, Schwartzman J, Augustin R, **Zhou L**, Ruby EG, Hourdez S, McFall-Ngai MJ (2014) The dual nature of hemocyanin in the establishment and persistence of the squid-vibrio symbiosis. *Proc. R. Soc. Lond. B* 281:20140504 | <http://dx.doi.org/10.1098/rspb.2014.0504>

Schwartzman J, Koch E, Heath-Heckman EAC, **Zhou L**, **Kremer N**, McFall-Ngai MJ, Ruby EG. (2015). The chemistry of negotiation: rhythmic, glycan-driven acidification in a symbiotic conversation. *PNAS* 112:566-571 | <https://www.doi.org/10.1073/pnas.1418580112>

Rader B, **Kremer N**, Apicella M, Goldman W, McFall-Ngai MJ (2012). Modulation of symbiont lipid A signaling by host alkaline phosphatases in the squid-vibrio symbiosis. *mBio* 3(3):e00093-12. <https://doi.org/10.1128/mBio.00093-12>

Belcaid M, Casaburi G, McAnulty SJ, Schmidbauer H, Suria AM, Moriano-Gutierrez S, Pankey SM, Oakley TH, **Kremer N**, Koch EJ, Collins AJ, Nguyen HLS, Goncharenko-Foster I, Minx P, Sodergren, E, Weinstock G, Rokhsar DS, McFall-Ngai MJ, Simakov O, Foster JS, Nyholm SV. 2019. Symbiotic organs shaped by distinct modes of genome evolution in cephalopods. *PNAS*. 116(8):3030-35 | <https://doi.org/10.1073/pnas.1817322116>

### 2.3 - Discussion

These results point the fact that heterogeneity within a host occurs not only in horizontally acquired symbioses, but also in vertically transmitted symbioses (Chrosteck & Teixeira 2015; Martinez & Sinkins 2023). Such heterogeneity could result from genomic rearrangements (e.g., duplications, recombinations, inversions, deletions) that can now be studied by genomic approaches at the scale of the individual life or organ, and open a new area on the study of rapid evolution.

If variations in genotypes are associated to changes in density, heterogeneity within a host raises the question of the selection level: in case of host determinism, does the host control the overall symbiont population by decreasing symbiont abundance independently of the symbiont genetic specificity, or does it target specific variants? In case of bacterial determinism, do variants compete each other by direct or indirect mechanisms, or are all variants counter-selected if their overall density impacts too much host fitness? Using proxies that mirror heterogeneity and/or specific staining associated with *in vivo* imaging could help addressing these questions. Parallel evolution could also help defining candidate genes involved in density regulation (Duarte *et al.* 2021; Martinez & Sinkins 2023). More generally, it seems important to consider both between-host and within-host selection when studying control and maintenance in symbioses.

Interestingly in both systems, bottlenecks play an important role on density and diversity of the symbiotic population, but with very different outcomes. In the vertically transmitted symbiosis between *D. melanogaster* and *Wolbachia*, transmission through the eggs decreases *Wolbachia* effective population size and strongly limits selection efficiency and potential adaptation processes associated with symbiont density. This result differs from studies carried out on mosquito cell lines with larger *Wolbachia* effective population sizes, in which mutations associated with changes in density were selected for (Martinez & Sinkins 2023). On the opposite, in the horizontally transmitted symbiosis between *E. scolopes* and *V. fischeri*, bottleneck in the light organ is rather associated with a 'sorting' and 'selection' mechanism that favors host-symbiont specificity and stability (Wollenberg & Ruby 2009).

A final interesting point highlighted by these studies is that symbiotic densities change over the course of individual's life (*e.g.*, variation along the day/night cycle, increasing density with age), whether these symbioses are mutualist or not. Control or tolerance mechanisms (*e.g.*, immune systems such as PGRP, AP or LBP, or metabolic regulation through access to substrates) have therefore probably evolved to cope with variations in symbiont density. In symbioses providing a benefit to the host, the intensity of selection of these regulation mechanisms may be stronger or more specific to dedicated organs such as light organs. Comparing immune systems in symbiotic and non-symbiotic organs could help defining control mechanisms that are shared or specific between mutualists and opportunistic pathogens.



## Chapter 3 – Multiple interactions & impact on the extended phenotype

Considering a symbiotic community raises the question of the role of interactions between organisms in establishing the extended phenotype. In this context, multipartite interactions occur between the host and its different symbionts, but also between microbial symbiotic communities.

Canonical examples of multipartite interactions impacting the host extended phenotype are **nutritional symbioses** that rely on multiple partners. Numerous examples have been described in insects feeding on unbalanced diets (*e.g.*, phloem, blood, grains...): *Baumania* and *Sulcia* respectively provide vitamins and cofactors or essential amino acids to sharpshooters, establishing a codependency between the three partners (Wu *et al.* 2006); the invasive tick *Hyalomma marginatum* also now relies on *Francisella* for the biosynthesis of folate, riboflavin and on *Midichloria* for the biosynthesis of biotin that are absent from their blood meals (Buysse *et al.* 2021). Interaction between bacterial partners can also play a major role in nutritional symbioses. In the fruit fly for instance, both *Acetobacter pomorum* and *Lactobacillus plantarum* promote larval growth (Consuegra *et al.* 2020), but a cross-feeding is needed under unbalanced diet. Indeed, *A. pomorum* uses the lactate produced by *L. plantarum* and supplies the latter in amino acids necessary for its growth (Henriques *et al.* 2020).

**Protective or defensive symbioses** between a mutualist partner and a pathogen or a parasite are another type of multipartite interactions impacting the host extended phenotype (Brownlie & Johnson 2009; Flórez *et al.* 2015; Vorburger & Perlman 2018). Different mechanisms have been selected for in the defensive symbiont to block pathogen/parasite load (*i.e.*, blocking) and/or to reduce its deleterious fitness effect on the host (*i.e.*, protection):

- 1) to compete for similar resources (*e.g.*, nutrients, membranes) or for the use of cellular machinery;
- 2) to produce chemical/physical compounds that directly 'attack' pathogens or parasites;
- 3) to prime host immune defense, and thus increase host resistance or tolerance;
- 4) to improve host fitness by the provision of nutrients, leading to a better defense against pathogens.

The study of multipartite interactions is quite recent and many mechanisms underlying changes in host extended phenotype are still unknown. In addition, these studies generally consider either interactions within a context of nutritional symbiosis or of protective symbiosis, but the simultaneous impact of both types of symbioses is not yet studied. These complex and dynamic interactions between host and multiple symbionts and their impact on life-history traits are challenging to study because they require a very controlled model system, where different combinations of interactions can be experimentally established. In mutualistic symbioses where adaptation led to an evolve dependence between partners, it is almost impossible to tease apart the influence of each partner, as the removal of one partner can induce a reduction in host performance that limits its survival or reproduction (de Mazancourt *et al.* 2005; Hammer 2023).

As part of my research, I thus chose to study the fruit fly *Drosophila melanogaster*, whose nutritional and protective symbionts are facultative and can be manipulated. In this chapter, I will describe two cases of multipartite interactions, their impact on host life history traits and the potential mechanisms involved in these phenotypes. The first one will only focus on the protective symbiosis conferred by the endosymbiont *Wolbachia* against the *Drosophila C virus* (DCV), while the second will focus on the multipartite interaction between two commensal nutritional gut symbionts, the endosymbiont *Wolbachia* and DCV.

### 3.1 - *Wolbachia* and DCV

*Wolbachia pipientis* has been considered for a long time solely as a reproductive parasite in arthropods, as its strains can induce cytoplasmic incompatibility, feminization, parthenogenesis or male-killing (Werren *et al.* 2008). However, in some species such as in *D. melanogaster*, the weak intensity of cytoplasmic incompatibility induced by wMel was questioning its large prevalence in natural populations. A factor that could explain its spread is its protective effect against various viruses, described in 2008 (Teixeira *et al.* 2008; Hedges *et al.* 2008). Since then, several studies have investigated its blocking or protective effect in various insects, in particular the spectrum of targeted pathogen, the *Wolbachia* strain/host specificity, and the mechanisms in play to explain such large panel of interference (reviews in Lindsey *et al.* 2018; Pimentel *et al.* 2021). *Wolbachia* density seems to play a major role in the efficiency of protection (Martinez *et al.* 2014; Chrostek & Teixeira 2015) and could play a blocking/protective role by competing with viruses for cellular and metabolic resources (Caragata *et al.* 2013, 2014; Geoghegan *et al.* 2017; Jiménez *et al.* 2019; Merklings *et al.* 2019; Lindsey *et al.* 2021). Immune priming seems restricted to *Wolbachia*-insect associations that do not have coevolved (Rancès *et al.* 2013), but cellular stress or active suppression/modification of viral particles cannot be ruled out in mosquitoes (Bian *et al.* 2013; Bhattacharya *et al.* 2020). It thus appears that many mechanisms could act together and vary depending on the host and the *Wolbachia* strain involved. However, all studies presented above used a route of infection which is not natural (*i.e.*, pricking), even though the route of infection of pathogens, either systemic (*i.e.*, pricking) or oral (*i.e.*, feeding), can influence the host immune response against bacteria (Gupta *et al.* 2017) or viruses (Ferreira *et al.* 2014). In addition, most studies focus on the adult stage and the protective effect of *Wolbachia* has not been much investigated during the whole life cycle.

In this context, I supervised the PhD project of Alexis Bénard, during which we established the *Drosophila/Wolbachia/DCV* model system in the lab, and in particular set up the experimental condition for natural infection<sup>12</sup>. We first characterized the effect of *Wolbachia* on various fly life history traits after DCV ingestion, and developed transcriptomic and metabolomic approaches to address potential mechanisms at play.

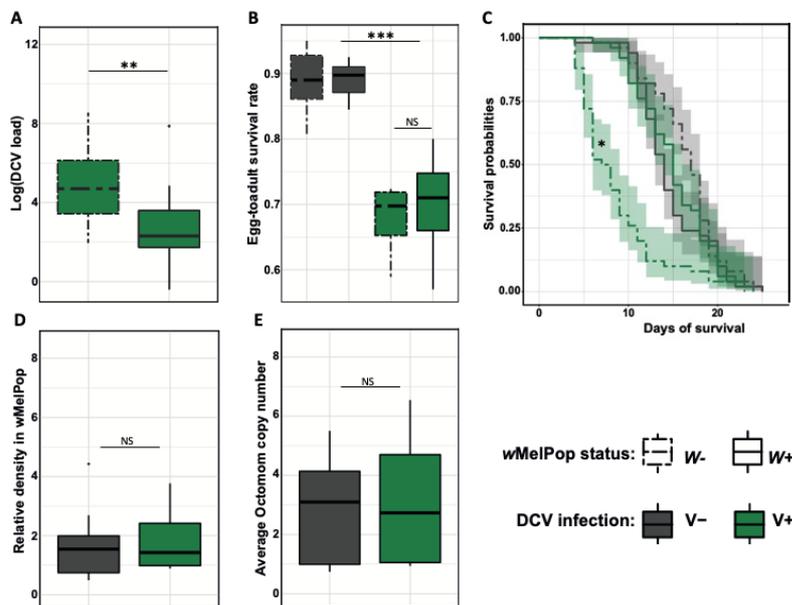
As observed in Lautié-Harivel (1992), DCV administrated by feeding larvae on DCV-infected cadavers during development allowed its persistence into the adult stage (**Figure 14A**), in contrast to DCV administrated twice as a solution at the L1 stage that was cleared after emergence (Mondotte *et al.* 2018). DCV can be pathogenic on larvae (**Figure 14B**) as well as on the resulting imagoes (**Figure 14C**), when the larvae were fed with infectious dead flies. We often observed diphasic survival curves, with a higher mortality rate during the 15 days following hatching. These results suggest that flies which survived after this period either cleared the infection (Duneau *et al.* 2017) or tolerated it.

While DCV induced larval mortality, we did not observe any protective effect of *Wolbachia* in larvae (**Figure 14B**), contrary to what was observed in Stevanovic *et al.*, 2015. However, the presence of *Wolbachia* protected adult flies that fully developed on a DCV-infected medium (**Figure 14C**), in the same way that it protects adult flies infected systemically by pricking (Teixeira *et al.* 2008; Chrostek *et al.* 2013; Martinez *et al.* 2015). In addition, this protection was mediated by a blocking of DCV replication in early adulthood (*i.e.*, resistance, **Figure 14A**). Inversely, the presence of DCV since the

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<sup>12</sup> Flies were pricked by DCV and let age on rich food medium to let the phenotype express. They were collected immediately after their DCV-induced death and frozen at -80°C. During experiments, these “dead” flies were deposited in the fly food, where they were ingested by developing fly larvae during their development. A dose of 6 flies (3 males and 3 females) was determined to optimize both mortality and *Wolbachia*-induced protection.

larval stage did not impact *Wolbachia* density or diversity (estimated here through the number of Octomom copies ; **Figure 14D,E**) two days after emergence.



**Figure 14 | Effect of DCV ingestion on *Drosophila* and *Wolbachia* life-history traits.**

**A.** DCV load (log) after ingestion in *D. melanogaster* flies harboring (full line) or not *Wolbachia* (dashed lines). **B-E.** Various host and bacterial life history traits measured in control (grey) or DCV ingestion (green) conditions: survival rate from egg to adult (**B**), probability of adult survival after emergence (**C**), relative density of *Wolbachia* (**D**), and average copy number of Octomom per *Wolbachia* within flies (**E**).

Infection by DCV was established by adding 6 cadavers of DCV-infected flies in the rearing medium. n = 10 flies / replicate line; 10 repl. lines / condition. Figure from Alexis Bénard.

We then explored the potential mechanisms involved in DCV infection and protection, using a combination of approaches: Dual-RNAseq in the gut was designed to capture variation in both host and bacterial gene expression, while global metabolomics in whole flies was designed to capture changes in metabolism and physiology. Although the Dual-RNAseq pilot experiment we carried out with H el ene Henri and the IGFL genomics platform<sup>13</sup> was very promising, we did not obtain the expected quantity of bacterial reads when we outsourced the service to IGATech (Italy) for all our samples. This problem unfortunately led us to analyze only reads from *Drosophila*. Because flies started to die from 4 days after adult emergence in the ‘DCV condition’, we chose to sample 2 days after adult emergence to avoid a bias sampling in surviving flies. In addition, we had noticed at the adult stage that infection patterns were following two specific trajectories, in accordance with the bifurcation curves observed for bacterial infection (Duneau *et al.* 2017): either DCV load reached very high densities and flies died within 5-10 days, or DCV infection was ‘cleared’ and flies survived more than 11 days.

Phenotypes induced by DCV on host life-history traits were detectable on larvae and adults, but surprisingly, the number of genes that were differentially regulated upon DCV ingestion were very low 2 days after adult emergence (14 (0.1%) up and 4 (0.04%) down), and those modulated by *Wolbachia* were absent. The small number of DE genes could be explained by different hypotheses: 1) DCV, when ingested, does not induce many transcriptomic changes in the gut; 2) metamorphosis could ‘reset’ DCV load to low levels, limiting major transcriptomic changes; 3) larvae with a high DCV load could have died before adult emergence and emerging adults could thus have low DCV infection load shortly after emergence, leading to a small transcriptomic response from the host side. All three hypotheses could occur, based on preliminary experiments on the kinetics of DCV infection over the whole life cycle (Lou Guyot L3 internship). The results show that DCV load was higher in larvae and pupae compared to

<sup>13</sup> Technology Nugen Ovation® using specific probes for the ribodepletions (‘universal’ prokaryotes + *Wolbachia* + *Drosophila* rRNA)

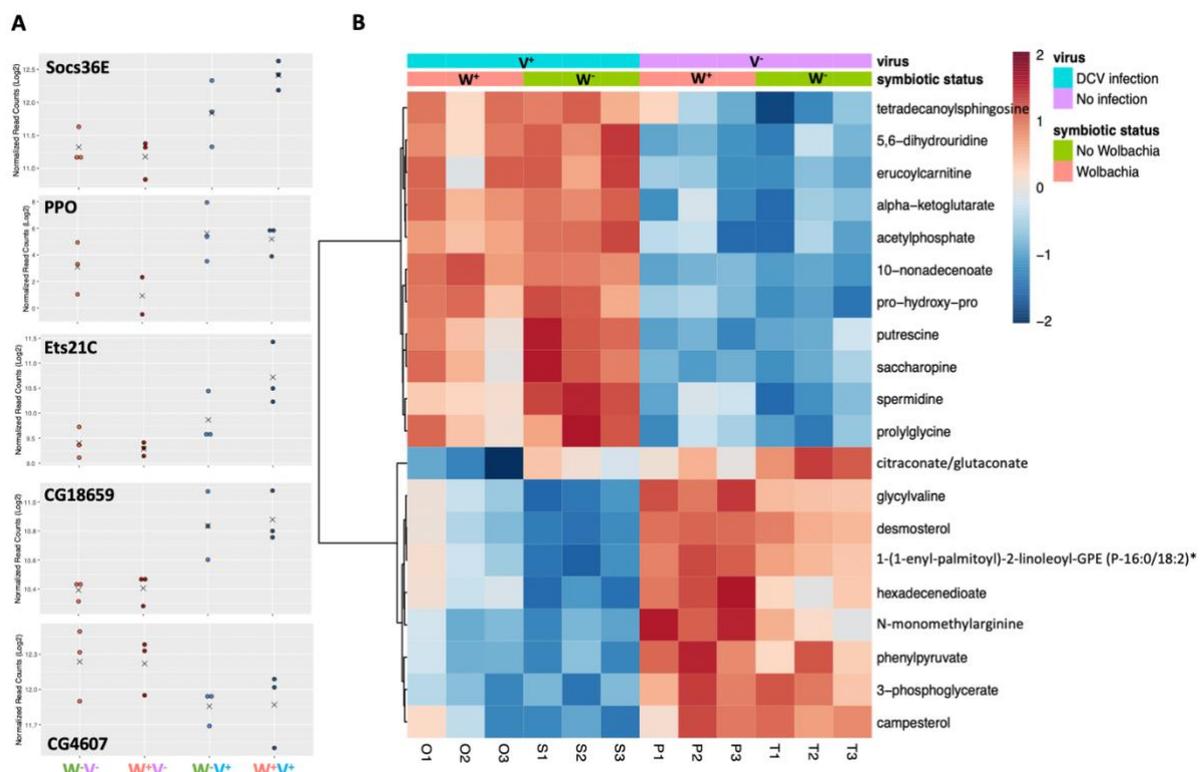
freshly hatched flies (day 0 to day 5) and that DCV load started to increase again in *Wolbachia*-free flies after 8 days post hatching.

While there were not many candidates upon DCV ingestion, we still found some interesting ones that may require further investigation. In particular, we detected potential immune regulators (**Figure 15A**):

- **Socs36E** (Suppressor of cytokine signaling at 36E), which is a negative regulator of the JAK/STAT pathway, known for its anti-viral activity, and already described to be derepressed upon DCV injection (Merkling *et al.* 2015);
- **Ets21C**, a stress-inducible transcription factor upon infection or oxidative stress, promoting tissue renewal in the midgut, upregulated upon early response to DCV in *Drosophila* S2 cells (Zhu *et al.* 2013);
- **PPO2**, a prophenoloxidase whose activity is known to play a role in immune defense against arboviruses in mosquitoes (Rodriguez-Andres *et al.* 2012);
- **CNT2** (Concentrative nucleoside transporter 2), which has a nucleoside : sodium symporter activity, and which could play a role in dSTING-dependent antiviral activity dependent in *Drosophila* enterocytes through cyclic dinucleotides (Segrist *et al.* 2021);

but also genes involved in metabolism, such as:

- **CG4607**, an orthologous gene to the human solute carrier family 2-member 6/8, involved in sugar transport. This gene exhibits a role in dietary sugar sensing in the midgut, potentially regulating lysosomal glucose metabolism, and its knockdown leads to fly lethality under high carbonate diet (Francis *et al.* 2021);
- **CG18659**, predicted to be involved in lipid binding.



**Figure 15 | Effect of DCV ingestion on *Drosophila* transcriptome and metabolome.**

**A.** Normalized expression of candidate differentially expressed genes based on DEseq2 analysis (3 replicate pools of a mix of 10 replicate lines /condition, the cross represents the mean). **B.** Heatmap of the 20 first hits from an ANOVA2 analysis ([metabolite] ~ DCV x *Wolbachia*, p-value after FDR correction > 0.01). The red-blue continuum indicates the differential of representation of each metabolite in each replicate (3 replicate pools of a mix of 10 replicate lines / condition; MetabolAnalyst 5.0).

In parallel, we sampled whole females to perform global untargeted metabolomics related to the metabolism of amino acids, carbohydrates, nucleotides, lipid, cofactors & vitamins, and metabolites related to energy and xenobiotics (Metabolon®, USA). 10% of the metabolites were differentially represented in response to DCV ingestion, among which 15% were also differentially represented in response to *Wolbachia* status (but no significant antagonistic interaction was detected). For more visibility, a heatmap (Figure 15B) shows the 20 most differentially represented metabolites in response to DCV and *Wolbachia* infection status.

The enrichment analysis mainly highlighted the over representation of metabolites involved in phenylalanine, polyamine (arginine & proline), pyrimidine and xanthine metabolism, but also a perturbation of the TCA cycle and the oxidative phosphorylation, and the involvement of dipeptides. Interestingly, polyamines (spermidine and putrescine), which are more concentrated after DCV ingestion, could play a role in virus infection (Firpo & Mounce 2020). Itaconate, which is produced in the mitochondria and turns down the TCA cycle, its isomere citraconate/glutaconate, and similar metabolites such as malonate also exhibit immune-metabolic properties in humans (Peace & O'Neill 2022), especially against Zika virus. In our system however, their concentration is decreased after DCV ingestion and in *Wolbachia*-infected flies.

Many lipids were also differentially regulated in response to DCV ingestion: sterols (desmosterol and campesterol) and lipids involved in carnitine metabolism and in fatty acid synthesis were down represented, whereas long-chain polyunsaturated fatty acids (n3 and n6), sphingosines and lipids involved in fatty acid metabolism (acyl-carnitine) were up represented. Lipids can play an important role in insect immunity (Wrońska *et al.* 2023). One interesting candidate is demosterol, whose concentration is reduced after DCV ingestion but increased by the presence of *Wolbachia*. This molecule, known to play a role during the hepatitis C infection by increasing lipid bilayer fluidity (Costello *et al.* 2016), could participate to the remodeling of lipid membranes during viral replication. Modulation of acyl-carnitines has been proposed to be a mechanism involved in *Wolbachia*-mediated blocking in the mosquito *Aedes aegypti* against flaviviruses such as Dengue virus or Chikungunya virus (Manokaran *et al.* 2020), but in our fly system, acyl-carnitines do not seem to be strongly impacted by the presence of *Wolbachia*.

This work is on progress and requires an in-depth analysis to determine more precisely the direct impact of an ingestion of DCV on gene expression in the gut and on metabolite concentration in the whole body, as well as the potential impact of *Wolbachia* on these phenotypes. Considering transcriptomics and metabolomics data together and performing network analyses could help provide an integrative view of the tripartite interaction between *Drosophila*, DCV and *Wolbachia*.

### 3.2 - Digestive microbiota, *Wolbachia* and DCV

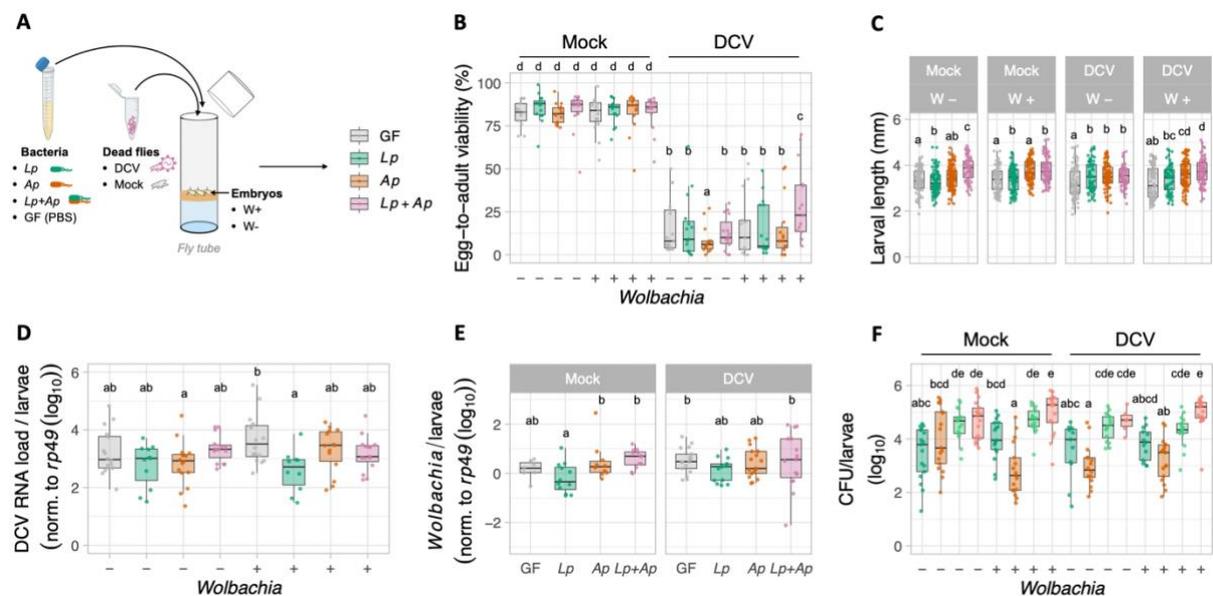
I then added another layer of complexity by considering the commensal gut community in addition to *Wolbachia*. Indeed, the impact of the interaction between endosymbiotic and commensal bacteria on viral infection was not explored yet.

Fruit flies are frequently associated with two main intestinal commensal bacteria, *Lactobacillus plantarum* and *Acetobacter pomorum*: *L. plantarum* is a major modulator of host metabolism in the juvenile stage, and recapitulates the growth-promoting effect of the classic *Drosophila* intestinal bacterial flora (Storelli *et al.* 2011); *A. pomorum* modulates energy metabolism and final body size through the insulin signaling pathway (Shin *et al.* 2011). Current knowledge suggests that *Wolbachia*-mediated virus blocking in insects is partly mediated by the modulation of host metabolism (Caragata *et al.* 2013, 2014; Geoghegan *et al.* 2017; Jiménez *et al.* 2019; Merklings *et al.* 2019; Lindsey *et al.* 2021).

The interaction between *Wolbachia* and these intestinal bacterial species could thus influence host physiology and potentially the response to viral infection. Bacterial symbionts can also provide antiviral protection. Indeed, intestinal commensal bacteria can interfere with viral infection (Ramirez *et al.* 2014; Sansone *et al.* 2015), and *Wolbachia* can also interact with the native microbiota, influencing the load and transmission of other bacterial symbionts in arthropods (Dittmer *et al.* 2014; Ramirez *et al.* 2014).

Within this context, I aimed together with Vincent Raquin (recruited post-doc) and François Leulier (IGFL Lyon) to decipher the interactions between intestinal commensal bacteria, *Wolbachia* and DCV in the model organism *D. melanogaster*. In the ComEndoVir project funded by the labex Ecofect, we thus tried to better understand how these interactions shape the outcome of viral infection through their effects on life-history traits into adulthood.

We first set up an experimental system comprising axenic individuals and gnotobiotics flies (*i.e.*, mono-associated with *L. plantarum* or *A. pomorum*, or bi-associated with both strains), colonized or not with *Wolbachia*, and then performed an oral infection (ingestion) with the DCV virus (Figure 16A). We then quantified various larval life-history traits, as well as viral and bacterial loads (Figure 16).



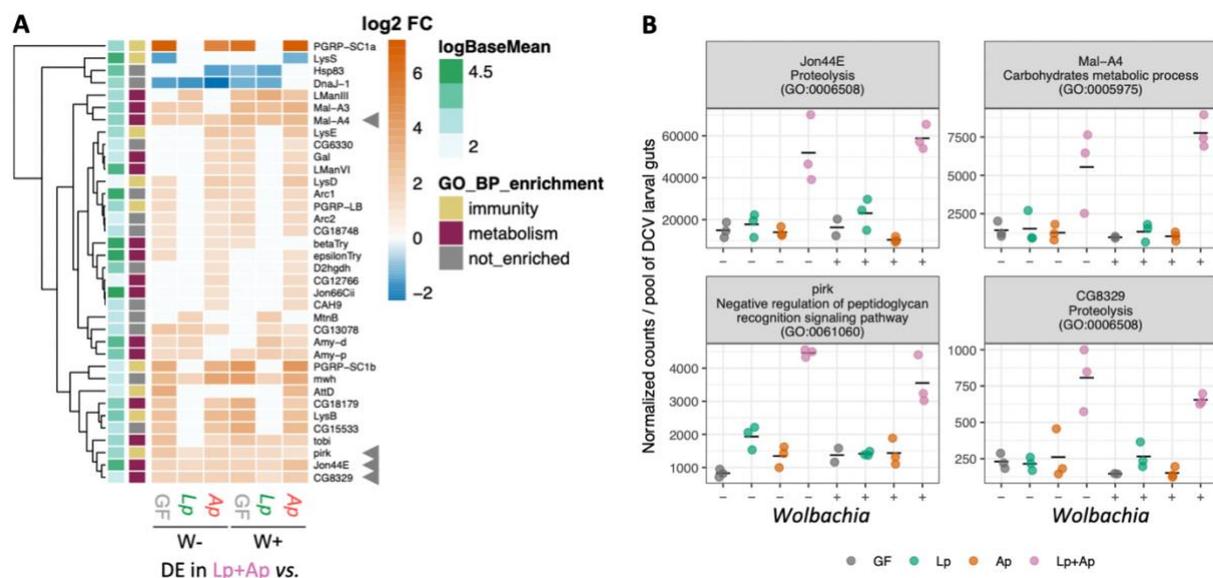
**Figure 16 | Effect of *Wolbachia* and digestive microbiota on fly larval response to DCV ingestion.**

**A.** Experimental protocol testing the effect of *Wolbachia* and gut microbiota (*Lp*: *Lactobacillus plantarum*; *Ap*: *Acetobacter pomorum*) on the response of larval flies to DCV ingestion. **B-C.** Measurement of life-history traits (**B**: Egg-to-adult viability; **C**: larval length) in mock or DCV infected flies, under different conditions of symbiotic association. **D-F.** Relative load of different microbes (**D**: DCV, **E**: *Wolbachia*, **F**: gut microbes) under different conditions of association. Letters above boxplots indicate statistical significance between conditions (*i.e.*, conditions with a different letter are statistically significant with a p-value < 0.05, ANOVA and Tukey post-hoc pairwise comparisons). Figures from Vincent Raquin.

We confirmed the pathogenic effect of DCV on larval development, and showed a significant effect of the combination of commensals and *Wolbachia* on egg-to-adult survival in a context of DCV infection (Figure 16B). This difference in survival could not be explained by a significant decrease in DCV load (Figure 16D) nor an increase in *Wolbachia* density (Figure 16E), rather suggesting a better tolerance of DCV in bi-associated larvae. These bi-associated larvae exhibited a longer length (Figure 16C), proxy of larval physiology, which could be explained by a higher larval gut content (data not shown) and a higher commensal density (Figure 16F). Hence, a higher ‘vigor’ of bi-associated larvae could lead to a better defense against pathogens.

When then defined transcriptomic changes associated to the higher resistance to DCV in bi-associated larvae. For this purpose, we extracted RNA from pools of 10 larval guts and sequenced cDNA using the Illumina technology. We then performed bioinformatics analyses and extracted genes that were differentially expressed (DE) between bi-associated larvae and either Germ-free or *Lp* or *Ap* mono-associated larvae (**Figure 17**). The gene ontology showed that most of DE genes were involved in biological processes related to immunity or metabolic processes. In particular, we isolated 4 genes that were commonly DE in all these comparisons, and could explain the fitness advantage of bi-associated larvae in presence of DCV:

- **Jon44E** encodes a serine-type endopeptidase, predicted to be involved in proteolysis, and shown to be over-expressed during larval development in presence of *L. plantarum*, and favoring juvenile growth promotion (Erkosar *et al.* 2015).
- **CG8329** encodes protein with a serine hydrolase activity also involved in proteolysis, and is down-regulated after sigma infection in females (Carpenter *et al.* 2009).
- **Mal-A4** encodes a maltase, protein involved in carbohydrate metabolic processes with a maltose alpha-glucosidase activity. This gene also appears to be up-regulated after the infection by a Nora virus (Cordes *et al.* 2013). Interestingly, alpha-glucosidases play a central role for the morphogenesis of many enveloped viruses (Chang *et al.* 2013). The presence of *Wolbachia* could also synergize the overexpression by *Ap+Lb* of other genes exhibiting a maltase activity (Mal-A3, and LManIII).
- **Pirk** encodes a negative regulator of the Imd pathway, acting at the level of product of PGRP-LC. It adjusts the Imd pathway activity to the severity of the infection (Kleino *et al.* 2008; Lhocine *et al.* 2008).



**Figure 17 | Effect of bi-infection versus mono-infection by *Lactobacillus plantarum* and *Acetobacter pomorum* on *Drosophila* larval transcriptome.**

**A.** Heatmap of the genes differentially expressed (DE) in bi-infected larvae compared to germ-free (GF) or mono-infected larvae, colonized (W+) or not (W-) by *Wolbachia*. The red-blue continuum indicates the differential of expression of each gene in each biological condition (n = 3 repl./cond.; 10 larval guts/replicate). The yellow/purple/gray colors indicate the biological process enriched after a Gene Ontology analysis. **B.** Normalized expression of candidate DE genes in bi-infected larvae. Figures from Vincent Raquin.

*Drosophila* genes involved in this multi-partite interaction could next be studied by *in vivo* functional analysis to confirm their involvement in promoting larval growth, but above all in their protective effect against DCV. This multidisciplinary project constitutes a proof of concept for characterizing the

influence of specific bacterial species (here *L. plantarum* and *A. pomorum*) on the *Drosophila* - *Wolbachia* - DCV interaction. In the longer-term, bacterial genera whose presence is modified by the presence of *Wolbachia* in wild larvae could be studied in a similar way. This study also highlights the importance of considering multi-partite interactions in the study of infectious diseases.

#### **Related publications:**

Raquin V, Galvão Ferrarini M, Saint-Michel E, Hughes S, Gillet B, Leulier F, **Kremer N**. Polymicrobial association promotes larval performance upon oral exposure to viral pathogen in an experimental *Drosophila* holobiont model. *In prep for ISME*

### 3.3 - Discussion

In this chapter, we studied the impact of symbionts on protection after ingestion of a natural fly virus. Contrary to the study from Mondotte *et al.* 2018, our protocol of DCV infection at the larval stage made DCV persist after fly emergence. The fact that we did not use purified DCV<sup>14</sup> but rather deposited DCV-infected cadavers (as in Lautié-Harivel 1992) suggests that priming of DCV inside the fly may be a necessary condition for making it more infectious or persistent in newly infected flies.

*Wolbachia* alone did not provide any significant protection in both studies in terms of egg-to-adult survival, and did not impact the viral load in larvae. In synergy with specific gut microbes however, *Wolbachia* did protect larvae, probably due to the positive impact of *L. plantarum* and *A. pomorum* on general vigor. *Wolbachia* alone did protect adult flies, by reducing the viral load 2 days post emergence in a rich medium with conventional microbiota (rich in *Acetobacter*), when the impact of microbiota on fly physiology is limited. Ye *et al.* 2017 found similar results after injection of DCV in adults, in which the changes in microbiota composition did not modify antiviral protection efficiency.

Finally, different strains of DCV have different tropisms for the digestive cells (Lautié-Harivel 1992), which may impair the efficiency of DCV infection and/or *Wolbachia* protection. Microscopic observations could thus indicate whether the localization of DCV and *Wolbachia* in host tissues differs between larval and adult stages and limits the tripartite interaction.

Compared to the intensity of the costs or benefits induced, transcriptomic and metabolomic analyses did not highlight many candidate genes linked to DCV antiviral response and to its interference with *Wolbachia*/microbiota. This small number of candidates could be due to the choice of the tissue (if responses are highly localized, performing analyses on whole larvae could dilute/compensate the tissue-specific signal) or the timing (if responses are development specific). In both cases, we were still able to detect a few candidate genes / metabolites linked to immunity and metabolism in response to DCV ingestion, suggesting that several immune pathways could be involved, and that 'non-canonical immunity' could play an important role. This 'non-canonical immunity' could be linked to an increased vigor of the host (at the larval stage) or to potential competition for nutrients. So far, attention was mainly focused on cholesterol, lipid, amino acids and nucleotide metabolism (Caragata *et al.* 2013, 2014; Geoghegan *et al.* 2017; Jiménez *et al.* 2021; Lindsey *et al.* 2021), but the study of carbohydrates could also be promising.

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<sup>14</sup> Preliminary attempts with cell produced DCV failed, even at very high doses.

More generally, studying multipartite interactions is rather complicated... If we want to consider all possible interactions, the characterization of the phenotype extended to several symbiotic partners is limited by resolution problems in meta-transcriptomics and meta-metabolomics, by the difficulty of identifying the origin of metabolites, the currency of interactions, by the difficulty of integrating data from several scales of study (*e.g.*, transcriptomics, proteomics, metabolomics, fluxomics, ...), by difficulties in spatial analysis and temporal dynamics. Developing tools for coupling taxonomic and functional models, methods for integrating interaction networks, multi-omics analysis methods and tools for studying the spatial structuring of communities into niches would help to overcome some of these obstacles.

We thus often limit the number of interactions that can be studied experimentally within a host to a few, and not all organisms can be cultivated. In this context, it is therefore useful to develop simplified or gnotobiotic experimental models (as realistic as possible), experimental twins or bioreactors. Functional genetics studies are progressing well in identifying the loci involved in the interactions between a few partners, notably with the use of deficient mutants and the comparison of their extended phenotypes with a wild-type strain. While intracellular bacteria such as *Wolbachia* are difficult to manipulate genetically, bacteria from the microbiota that can be used in gnotobiotic hosts can be more easily manipulated. Also, host genetic models such as *Drosophila* can now be more easily manipulated by RNAi or CRISPR technologies to validate host effectors involved in the interaction.

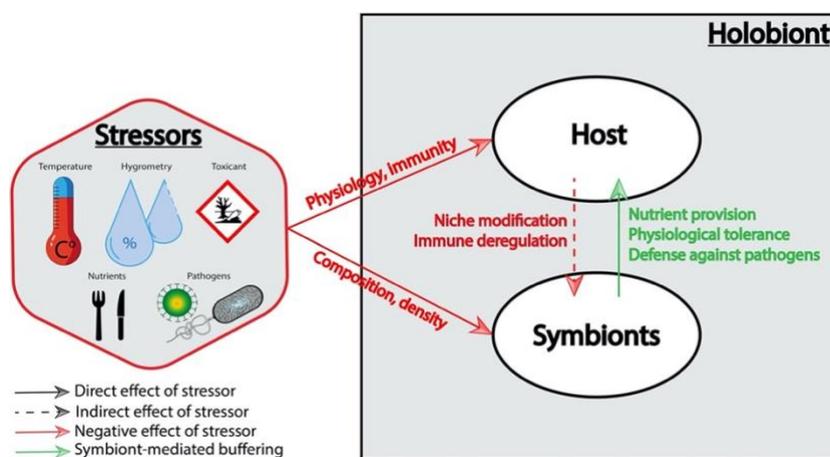


## Chapter 4 – Environment: impact of stressors

In nature, organisms live in changing environment. Indeed, they can face many **stressors**, defined here as any abiotic or biotic environmental factor able to disrupt homeostasis. Abiotic stressors include changes in temperature, water supply, nutrients availability, but also toxicants (pesticides, insecticides, chemicals...), whereas biotic stressors include infection by pathogens or parasites, predation... These stressors can induce a **stress response**, such as a change in physiology, immunity, behavior, but when the stress response is associated with a decline in the fitness of the organism, we will call it a **stress** (Schulte 2014).

Because organisms are always in symbiosis with other organisms, stressors will impact not only the host but also the associated symbionts, and will potentially impact directly or indirectly each partner and the holobiont (**Figure 18**, review in Bénard *et al.* 2020). For instance, a change in host physiology can modify the niche in which an endosymbiont resides, or a change in host immunity can impact the immune homeostasis that maintains the symbiont within the host at a defined density. When holobionts adapt to such changes through the selection of genetic or plastic mechanisms, the ability to persist is called **resilience** (Hodgson *et al.* 2015; Rymer *et al.* 2016). However, when it is not the case, stress may have critical outcomes on the symbiotic association, and induce a breakdown of the association and a perturbation of the ecosystem (Paxton *et al.* 2013; Putnam *et al.* 2017).

On the opposite, some symbionts can buffer environmental stressors by inducing a physiological **tolerance**, protecting against pathogens or providing nutrients that enhance host fitness (Clay 2014; Lemoine *et al.* 2020), and be considered as an adaptative stress response. However, because stressors can fluctuate in terms of frequency, amplitude and combination, but also because the transmission mode of the symbiont can limit its acquisition or maintenance within the host, we can wonder whether symbiosis is always the optimal strategy, that is, the more efficient one to buffer the stress at a reduced cost. In other words, if symbiosis drives or limits host adaptation to a new, stressful environment?



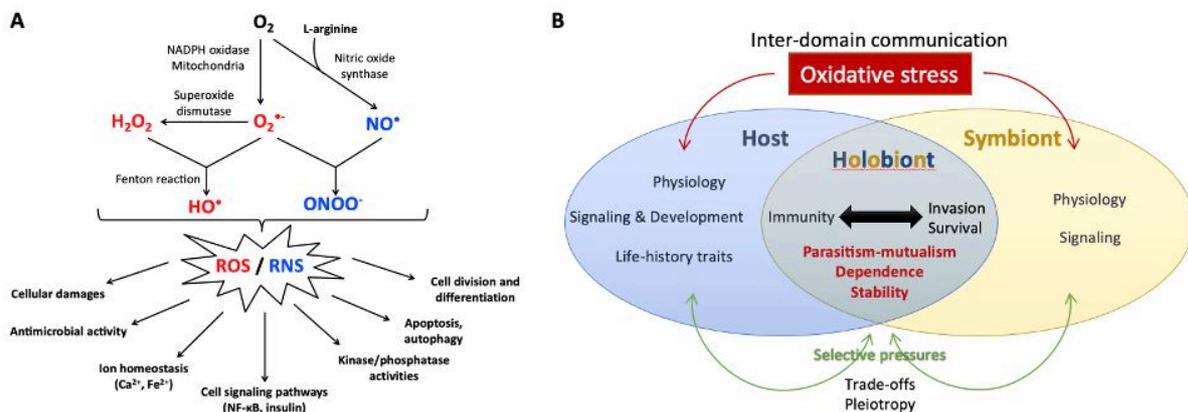
**Figure 18 | Effect of stressors on the holobiont.**

Stressors can directly alter the host physiology and immunity, and the symbiotic community. Stressors may also indirectly affect the symbiotic community by altering the host niche and the immune state of the host. Conversely, symbionts can buffer stressors *via* nutrient provision, physiological tolerance, and defense against host natural enemies. Figure & legend from Bénard *et al.*, 2020.

We will address this question by focusing on two main stressors in the *Drosophila-Wolbachia* symbiosis: oxidative stress (as a biotic stressor) and infection by a pathogen (as a biotic stressor). As part of the RESIST project (ANR funding), we aimed to examine the vulnerability of both partners, and to determine how these stressors impact (alone or in combination) hosts and symbionts. We also wanted to find out whether the symbiotic association would respond and potentially adapt to these

perturbations. Because the impact of DCV infection has been described in the previous chapter, and can be considered as an important biotic stressor, I will now focus on the oxidative stress and the relevance to study it in our research project.

The oxidative environment results from exogenous, environmental exposure to oxidizing compounds (nutrients, toxins, toxicants such as pesticides, herbicides...), but also from several chemical reactions within the cell, especially in the mitochondria and peroxisomes. **Reactive Oxygen Species (ROS)** and **Reactive Nitrogen Species (RNS)** strongly impact cell biology and physiology (**Figure 19A**), as they are involved in signaling pathways (*e.g.*, NF- $\kappa$ B, insulin, kinase/phosphatase activity) and basic cell functions (*e.g.*, cell division and differentiation, ion homeostasis, apoptosis, immunity). In the same time however, their reactivity can be deleterious and induce cellular damages (damages to DNA, proteins and carbohydrates; lipid peroxidation) and detoxification processes and feedback loops play a major role in limiting their toxicity (for detailed reviews on ROS and RNS, see Martínez & Andriantsitohaina 2009; Nathan & Cunningham-Bussel 2013; Imlay 2013; and Sies *et al.* 2022). Oxidative stress occurs when there is an “*imbalance of oxidants over antioxidants and repair processes, leading to a disruption of redox signaling and control and/or molecular damage*” (Sies *et al.* 2022). In a context of symbiosis, one major question is thus to determine how oxidative stress does impact cellular, physiological functions and life history traits of both host and symbionts, in particular those involved in immune regulation and survival. Also, we can wonder how oxidative stress and detoxification/tolerance mechanisms apply selective pressures that impact the symbiotic association itself, its position along the continuum between parasitism and mutualism, the level of dependency between symbiotic partners within the holobiont, and the stability of the association (**Figure 19B**, Moné *et al.* 2014).



**Figure 19 | The oxidative environment and its influence on biological functions and on the evolution of the holobiont.** **A.** Origin and pleiotropic effects of reactive oxygen (ROS) and nitrogen species (RNS): Superoxide anion ( $O_2^{\cdot-}$ ) is generated by mitochondrial oxidative phosphorylation reaction and by NADPH oxidase,  $O_2^{\cdot-}$  is then converted to  $H_2O_2$  by the superoxide dismutase enzyme,  $H_2O_2$  can further react with  $Fe^{2+}$  to produce hydroxyl radical ( $HO^{\cdot}$ ) by the Fenton reaction, Nitric oxide ( $NO^{\cdot}$ ) is synthesized from L-arginine by the action of nitric oxide synthase, and Nitric oxide can react with  $O_2^{\cdot-}$  to produce peroxynitrite ( $ONOO^{\cdot}$ ). ROS and RNS (RONS) modulate various cellular processes. **B.** RONS affect various biological functions of the host and the symbiont, thus influencing many aspects of their interaction. The resulting selective pressures, acting on one partner or at the holobiont as a whole, can drive the evolution of major characteristics of the symbiosis (influence the position of the association along the continuum between parasitism and mutualism, level of dependence between partners, stability of the symbiotic association). Figures & legends from Moné *et al.*, 2014.

I started to address these questions during my PhD, when I tempted to decipher the molecular bases of dependence between a parasitoid wasp, *Asobara tabida*, and one of its *Wolbachia* strain, wAtab3.

Indeed, the removal of *wAtab3* by an antibiotic treatment leads, depending on the host population, either to the absence of egg production or to the production of unfertile eggs (Dedeine *et al.* 2001; Kremer *et al.* 2010), and a strong apoptotic signal is observed in the developing ovaries (Pannebakker *et al.* 2007). We showed that *Wolbachia* strongly interferes with iron homeostasis, and that host compensatory mechanisms must have been selected for to limit perturbations induced by the presence of symbionts, leading to dependence between symbiotic partners (Kremer *et al.* 2009, 2012). We also showed that *Wolbachia* also interferes with iron metabolism in *Drosophila simulans* and in *Aedes aegypti* cells, suggesting a general effect of *Wolbachia* on iron homeostasis in its hosts (Kremer *et al.* 2009). These results suggest that iron homeostasis can play a major role in the physiology of both symbiotic partners and in extreme cases in the evolution of dependence.

Oxidative stress, and more specifically iron homeostasis, could thus play an important role in the ecological success of *Wolbachia* in several symbioses and the evolution of symbioses (Gill *et al.* 2014). Because the oxidative environment is impacted by the presence of toxicants and pathogens, we wanted to further explore its impact on symbiotic partners, and on the evolution of the symbiotic association under such selective pressure. I will thus present several studies focusing on the *D. melanogaster/Wolbachia* symbioses that we conducted on this topic.

#### **Related publications:**

**Kremer N**, Voronin D, Charif D, Mavingui P, Mollereau B, Vavre F. (2009) *Wolbachia* Interferes with Ferritin Expression and Iron Metabolism in Insects. *PLoS Pathogens* 5(10): e1000630 | <http://doi.org/10.1371/journal.ppat.1000630>

**Kremer N**, Dedeine F, Charif D, Finet C, Allemand R, Vavre F (2010) Do variable compensatory mechanisms explain the polymorphism of the dependence phenotype in the *Asobara tabida* / *Wolbachia* association? *Evolution* 64(10): 2969-79 | <https://doi.org/10.1111/j.1558-5646.2010.01034.x>

**Kremer N**, Charif D, Henri H, Gavory F, Wincker P, Mavingui P, Vavre F (2012) Influence of *Wolbachia* on host gene expression in an obligatory symbiosis. *BMC Microbiology* 12: S7 | <https://doi.org/10.1186/1471-2180-12-S1-S7>

Moné Y \*, Monnin D \*, **Kremer N** (2014) The oxidative environment: a mediator of inter-species communication that drives symbiosis evolution. *Proc. R. Soc. Lond. B.* 281: 20133112 (review) | <https://www.doi.org/10.1098/rspb.2013.3112>

Bénard A, Vavre F, **Kremer N**. 2020. Stress & Symbiosis: heads or tails? *Frontiers in Ecology and Evolution*. 8:167 | <https://www.doi.org/10.3389/fevo.2020.00167> (review).

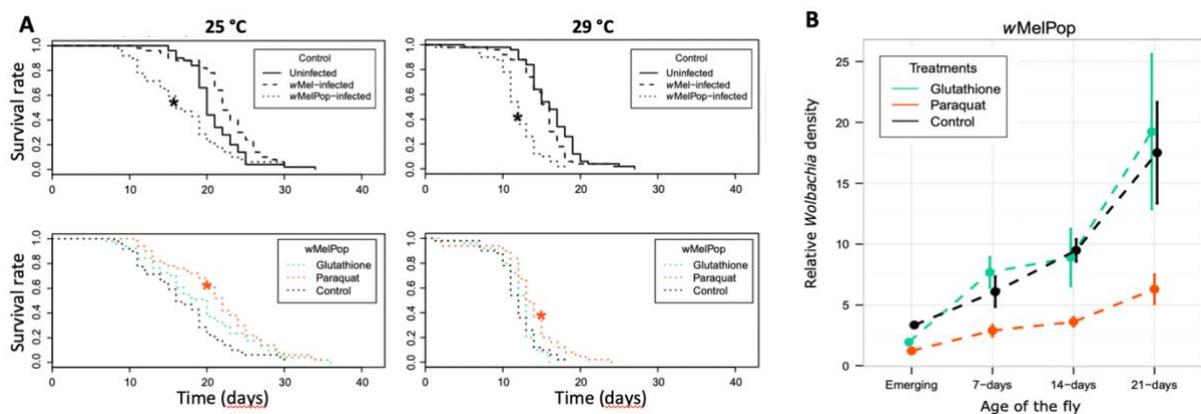
## 4.1 - Impact of oxidative stress on the *Drosophila-Wolbachia* symbiosis

### 4.1.1 - Direct impact of paraquat on the *Drosophila-Wolbachia* symbiosis

We first studied the impact of **paraquat** (N,N'-dimethyl-4,4'-bipyridinium dichloride), an herbicide that interferes with the chain of electron transfer and produces free radicals. It is frequently used in science to catalyze the formation of ROS, particularly the superoxide anion. Paraquat is banned in France and more generally in the European Union since 2007, because of its toxicity against human and animals,

but is still commercialized in the US, and in many countries in South America, Africa and Asia, making it a relevant toxicant that flies can face in nature.

During the PhD project of David Monnin, we first evaluated the direct impact of paraquat on *Drosophila melanogaster* and *Wolbachia* life history traits (Monnin *et al.* 2016). We chose to study the wMelpop *Wolbachia* strain, because of its high proliferation rate and its resulting cost, increasing with temperature. Based on its general inhibitory effect on many bacterial species among which *Wolbachia* (Peterson *et al.* 1981; Fallon *et al.* 2015), we expected a decrease in bacterial density following its application on fly diet at sublethal concentration, and thus a reduction in the cost of harboring wMelpop. We did demonstrate the pathogenic effect of *Wolbachia* on *D. melanogaster* starting above 25°C, and the positive impact of paraquat exposure on fly survival, linked to a reduction in *Wolbachia* density (Figure 20). Interestingly, a treatment with glutathione, known to detoxify ROS, did not impact significantly increase fly survival above 25°C, suggesting that, in control conditions, bacterial density induces a cost mainly independent of ROS production.



**Figure 20 | Influence of oxidative homeostasis on survival and bacterial density.**

**A.** Effect on fly survival of the *Wolbachia* infection status in control condition (top) and of the oxidative treatments in wMelpop infected flies (bottom). Black asterisks indicate a significant effect of the infection by *Wolbachia* compared to uninfected flies ( $p < 0.05$ ). Colored asterisks indicate a significant effect of treatments compared to control ( $p < 0.05$ ). **B.** Effect of oxidative treatments on *Wolbachia* (wMelPop) density (mean  $\pm$  SE). Figures and legend from Monnin *et al.*, 2016.

We confirmed the negative impact of paraquat exposure on *Wolbachia* density using *D. melanogaster* flies originating from different populations around the world (RESIST project), at a concentration that does not significantly impact larval and adult survival (4 mM, see data presented later on chapter 4.2.2).

During Camille Huot's Master 2 internship (co-supervised with Fabrice Vavre), we also tested the influence of host and bacterial genotypes on the regulation of oxidative homeostasis, and the impact of this regulation on symbiont density and host life-history traits. We chose the *Drosophila simulans* / *Wolbachia* model, in which numerous host and bacterial genotypes are available. This study shows that the host genotype had an impact on the density variations observed following modification of the oxidative environment, but with no repercussions on survival. Conversely, although bacterial densities were significantly reduced by a pro-oxidant treatment, the different bacterial genotypes had a similar response to changes in the oxidative environment. These results confirm the negative impact of oxidative stress on bacterial density in *Drosophila*, and suggest that the host genotype predominantly determines the density fluctuations observed in response to oxidative stress in *D. simulans*.

## Related publications:

Monnin D, Kremer N, Berny C, Henri H, Dumet A, Voituron Y, Desouhant E, Vavre F. (2016). Influence of oxidative homeostasis on bacterial density and cost of infection in *Drosophila-Wolbachia* symbioses. *Journal of Evolutionary Biology*. 29(6):1211-1222 | <https://doi.org/10.1111/jeb.12863>

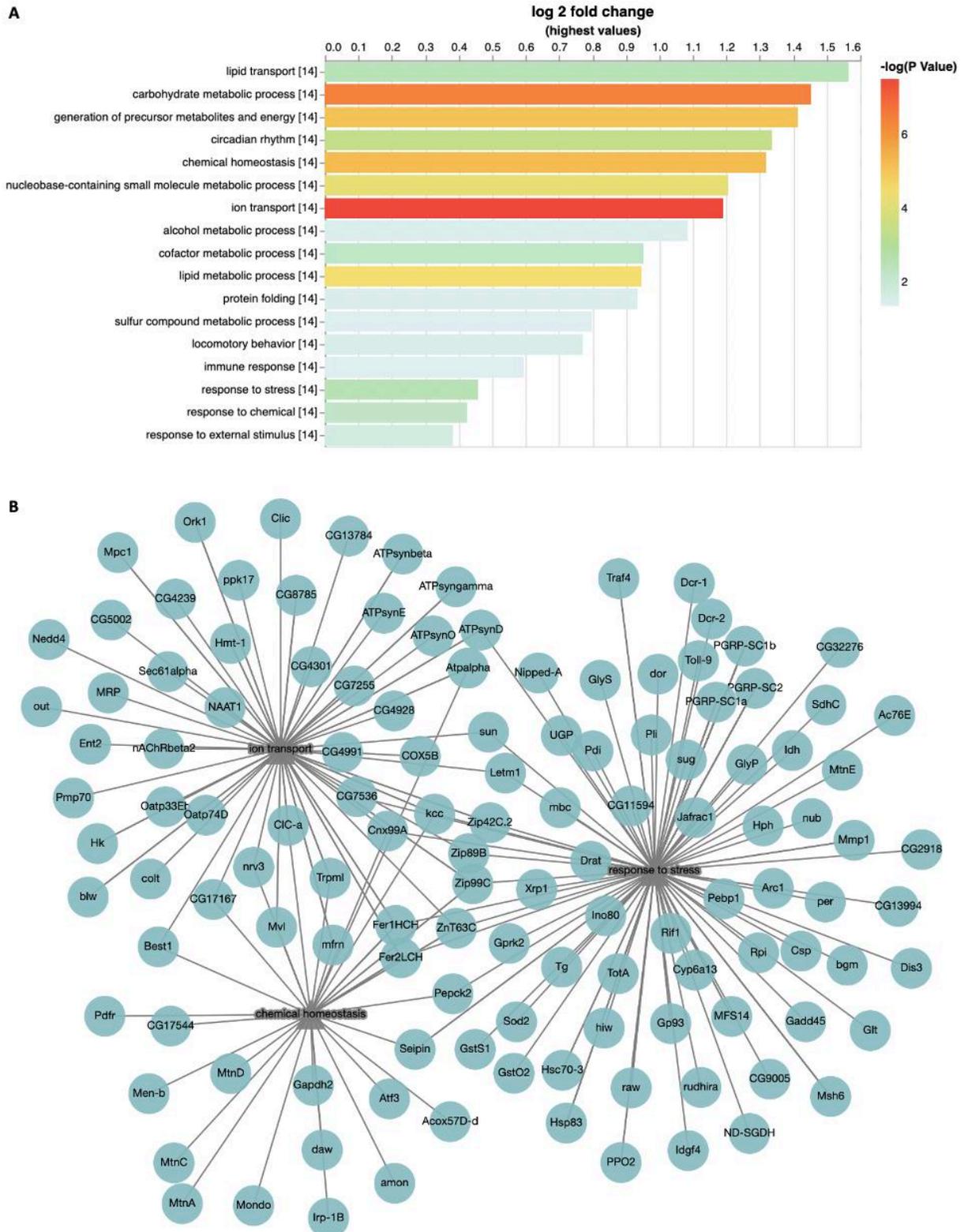
### 4.1.2 - Molecular mechanisms involved in response to paraquat

Similarly to the response to DCV infection (see [chapter 3.1](#)), we performed within the RESIST project transcriptomic and metabolomic analyses to compare gene/metabolite expression in response to paraquat ingestion (4 mM during the larval stage) and *Wolbachia* symbiotic status. In fly guts, 599 genes were differentially regulated in response to paraquat ingestion. Using the PANGEA enrichment tools (Hu *et al.* 2023), we extracted the enriched biological processes and related DE genes (SLIM-GO BP, see [Figure 21](#)).

We confirmed that paraquat did impact chemical homeostasis, ion transport and response to stress, and detected Ferritin or Zinc/iron regulated transporter-related proteins (Zip) involved in iron transport, but also detoxification genes such as Superoxide dismutase (Sod), Glutathione-S-transferase (Gst) or heat choc proteins. We also showed that paraquat ingestion impacted the respiratory chain and induced a large metabolic reprogramming including carbohydrate, lipid and cofactor metabolic processes, and an upregulation of immune genes. Among the genes DE in response to paraquat ingestion, whose expression varied according to *Wolbachia* infection status, we found only 4 transcripts encoding for: CG13936, a CNMamide encoding a short signaling peptide that shows a potent agonist activity toward the G-protein coupled receptor encoded by CNMaR; CG8745, an ethanolamine-phosphate phospholyase (Involved in response to nicotine); CG5150, an Alcaline Phosphatase; and CG15095, a Major Facilitator Superfamily Transporter (MFS14) involved in response to hypoxia. However, while some of these candidates could play a role in the response to stress (nicotine and hypoxia), they expressed a small log<sub>2</sub>-fold change and a p-value just under significance, and need to be considered with caution.

We then performed a metabolic analysis based on whole fly extracts and determined the influence of paraquat ingestion and the potential interference of *Wolbachia* with this process ([Figure 22](#)). As for changes in gene expression, we noticed a strong metabolic reprogramming (70/456 metabolites = 15.4 %). Based on pathway analysis (MetaboAnalyst 5.0), upregulations mainly included pyrimidine metabolism (glutamine, thymine, orotate, uracil, pseudouridine, 3-ureidopropionate), lysine degradation (lysine, saccharopine, 4-trimethylammoniobutanoate), and arginine / beta-alanine biosynthesis, but also certain classes of lipids (long-chain fatty acids, carnitine, inositol...). Interestingly, paraquat strongly decreased the representation of carbohydrate metabolism (in particular sucrose, fructose), and even more strongly in *Wolbachia*-free flies.

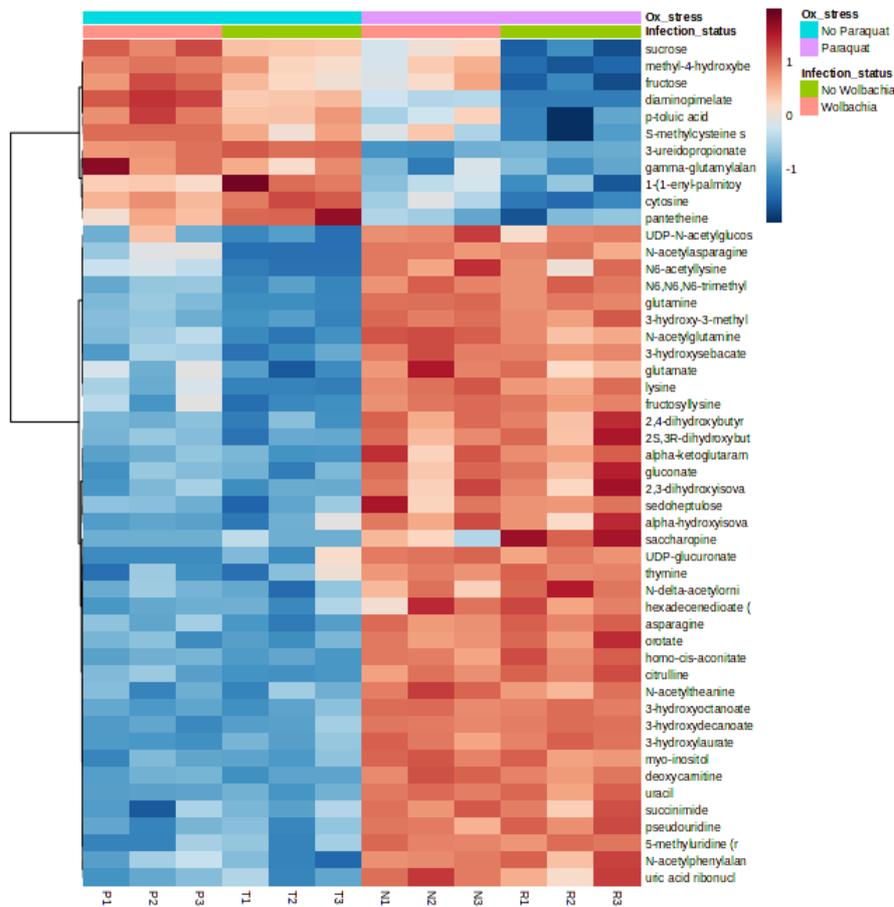
Paraquat is known to be reduced at both inner and out membranes of the mitochondria, and to impact mitochondrial lipid peroxidation, mitochondrial ROS, and to decrease Oxygen consumption, ATPase and activity (Blanco-Ayala *et al.* 2014). While the analyses presented above are preliminary and require an in-depth characterization both in terms of analytic methods (transcriptomics, metabolomics and integration of both approaches), biological pathways involved, and impact on the cell physiology or life history traits, they provide interesting candidates on the impact of the ingestion of a medium dose (but still sublethal) of paraquat during fly development.



**Figure 21 | Influence of paraquat 4 mM on fly gut transcriptome.**

**A.** Enrichment analysis resulting from DESeq2 analysis and PANGEA enrichment, using the Gene Ontology (Slim-Go Biological Processes; p-value threshold: 0.05; 3 replicate pools of a mix of 10 replicate lines /condition). Log<sub>2</sub> fold changes are indicated on the barplot, and p-values are color coded on the bars according to their value.

**B.** Differentially expressed genes (blue circles) associated with 3 selected DE biological processes (grey triangles: ion transport, chemical homeostasis and response to stress). Genes associated with 2 or 3 processes are connected by 2 or 3 nodes, respectively.



**Figure 22 | Influence of paraquat 4 mM on fly metabolome.**

Heatmap of the 50 first hits from an ANOVA2 analysis ([metabolite] ~ Paraquat x *Wolbachia*, p-value after FDR correction > 0.01). The red-blue continuum indicates the differential of representation of each metabolite in each replicate (n = 3 replicate pools of a mix of 10 repl.lines / condition; Metabolanalyst 5.0).

The large impact of paraquat on many metabolic processes is in accordance with previous studies on fruit flies (Girardot *et al.* 2004; Doran *et al.* 2017), which also suggest an efficient tolerance of flies against this stress. Interestingly, our study shows that *Wolbachia* could interfere with this stress response, especially at the metabolic level - through its influence on sugar availability-, and impact potential physiological (energy) or behavioral (flight) resources allowing flies to tolerate or resist to other stressors.

#### **Related publications:**

Bénard A, Jacquet A, Henri H, El Filali A, Galvão Ferrarini M, Vavre F, Kremer N. Transcriptomic and metabolomic impact of Paraquat ingestion in the symbiosis between *Drosophila melanogaster* and *Wolbachia*. *In preparation*.

#### 4.1.3 - Evolution of the *Drosophila-Wolbachia* symbiosis in response to oxidative stress

As presented before, the wMelPop strain of *Wolbachia* is particularly interesting to study, as it contains a genomic region whose number of repeats varies between bacteria, even within a host. This genomic region therefore provides a suitable tool for studying the action of selection at these different levels (within- and between-host), in response to stressful agents.

We know that *Wolbachia* density increases fly mortality (Min & Benzer 1997) and that paraquat decreases *Wolbachia* density (Monnin *et al.* 2016). We thus tested the following hypothesis concerning a potential impact of oxidative stress on the evolution of the symbiotic association: under experimental conditions where between-host selection may come into play (*i.e.*, late reproduction at a temperature (28°C) that induces pathogenicity of wMelPop), virulence should be reduced *via*

selection of variants with low Octomom copy numbers. On the contrary, virulence should not decrease in the presence of paraquat, which limits bacterial density and thus between-host selection. To test this hypothesis, we carried out an experimental evolution study over 17 generations with David Monnin (PhD co-supervised with F. Vavre and E. Desouhant). Contrary to our expectations, our results did not show a differential evolution of virulence between control and paraquat conditions, and on the contrary we detected an increase in symbiotic density and Octomom copy number in both conditions ([Monnin et al. 2021](#)). These results could be explained by the importance of within-host selection, reflected by the increase in density with age (see [chapter 2.1](#)). The results of this study underline that within-host selection could play an important role in the evolution of vertically transmitted symbionts, and could help explain the persistence of relatively virulent *Wolbachia* strains in nature.

#### **Related publications:**

Monnin D, Kremer N, Michaud C, Villa M, Henri H, Desouhant E, Vavre F. 2021. Experimental evolution of virulence and associated traits in a *Drosophila melanogaster* – *Wolbachia* symbiosis. *Peer Community Journal*, section *Evolutionary Biology* | <https://doi.org/10.24072/pcjournal.9>

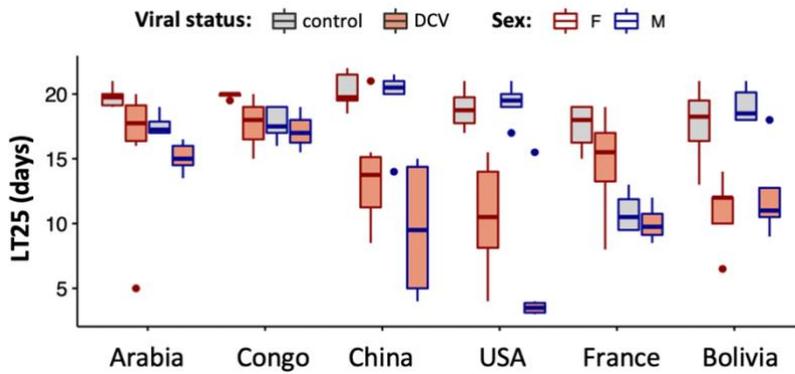
## 4.2 - Multiple stressors and evolution under chronic stress

In nature, stressors vary in intensity, frequency and persistence, and they rarely occur alone. Indeed, insect pests that thrive in agricultural fields are often exposed to insecticide or herbicide treatments, while also being subjected to attacks by pathogens or parasites. These observations raise many questions: (i) what happens when the two (or more) stressful agents occur at the same time? (ii) does the evolution under a persistent stressful condition impact the symbiotic association, and optimize or decrease its response to the same stressor? and (iii) does the evolution under a stressful condition impact the outcome of the exposure to another stressor? These questions are at the heart of the RESIST project, whose combined stress and experimental evolution approaches I will now describe.

### 4.2.1 - Set up of the experimental system

When we want to address the questions of evolution and adaptation, we need to work with an experimental system where variability is observed and heritable. From the host side, I chose to work with 6 populations of *D. melanogaster* that were initially uninfected by *Wolbachia* and that did not carry the DCV resistance alleles within the *Pastrel* gene ([Magwire et al. 2012](#); [Martins et al. 2014](#); [Martinez et al. 2019](#)). These populations had been trapped by lab members a few years ago around the world (Bolivia, China, Congo, France, Arabia, USA). From the bacterial side, my previous work on wMelPop showed a large variability in terms of Octomom copy number within lines and also within individuals. We thus backcrossed the 6 *Drosophila* natural populations with a w<sup>1118</sup> strain infected by wMelPop exhibiting a high average number of octomom copy numbers coupled with a strong variability between individuals ([Bénard et al. 2021](#)). For each line, we created an equivalent *Wolbachia*-free line, performing two successive rifampicin treatments and restoring the gut microbiota by seeding with male fly droppings.

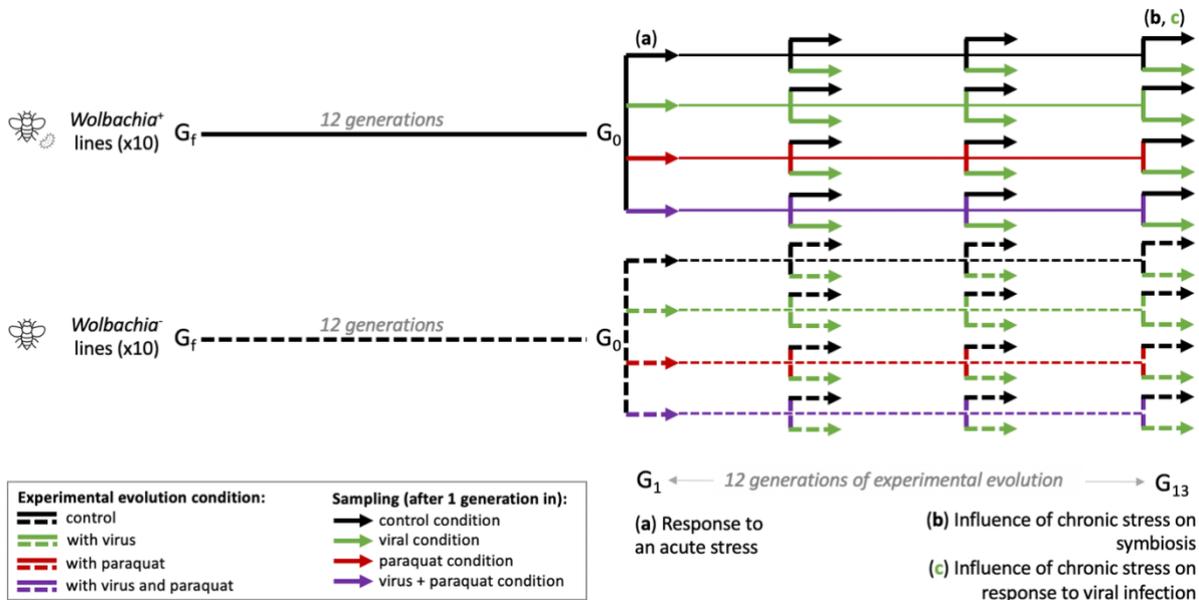
Because variability is well described concerning the response of *D. melanogaster* to paraquat ([Girardot et al. 2004](#); [Lovejoy et al. 2021](#); [Marin et al. 2021](#)), we only checked the variability of response to DCV in the backcrossed lines (Lou Guyot, L3 internship, **Figure 23**). These results do show variation in the survival rate after DCV infection according to fly population.



**Figure 23 | Influence of host genetic background on survival after DCV ingestion.**

Survival is indicated by the LT25 proxy, which indicates the number of days after which 25 % of individuals died. DCV ingestion occurred throughout the larval stage. F: Female, M: Male. ANOVA on log-transformed data: virus:  $p < 0.001$ ; population:  $p < 0.001$ ; sex:  $p < 0.001$ ; virus x pop:  $p < 0.01$ , pop x sex:  $p < 0.001$ ). Figure: Lou Guyot.

We then set up the following experiment (Figure 24): we applied an acute stress (DCV alone, Paraquat alone, DCV + Paraquat or control;  $G_1$ ) and let evolve the system under this stress regime for 12 generations. At the end of the experimental evolution ( $G_{13}$ ), we then tested the association under relaxed condition (to determine the impact of the evolution regime on the association) or under viral stress (to determine the impact of the evolution regime on the evolution of antiviral protection). These experiments were performed using a mix of the 6 backcrossed lines (10 replicate lines each), colonized or not by *Wolbachia*.



**Figure 24 | Experimental set-up to test the impact of acute stressors (alone or in combination, a) and of chronic stress exposures (b, c).** Plain lines correspond to *Wolbachia*-infected lines, whereas dashed lines correspond to *Wolbachia*-free lines. Females were allowed to lay eggs after 5 days post emergence, to let between-host selection occur (at least for the viral condition).

#### 4.2.2 - Impact of multiple stressors

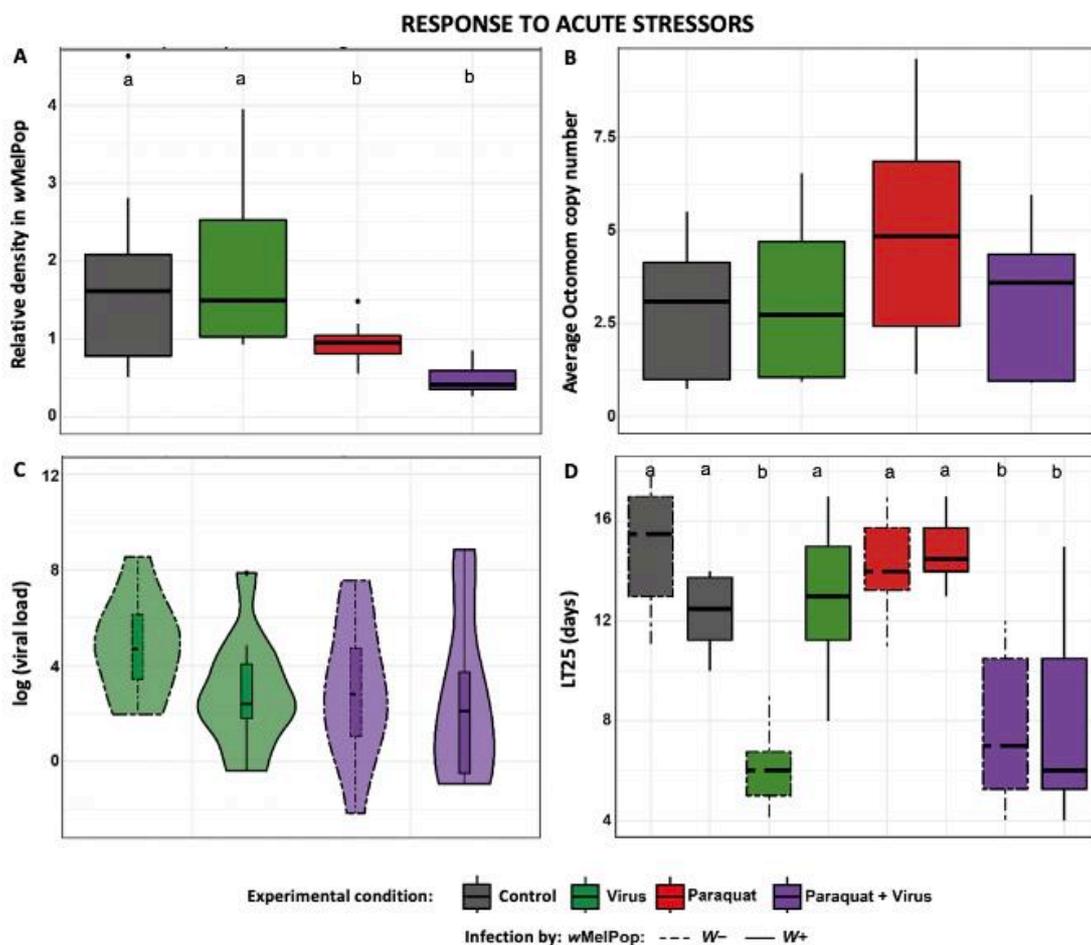
As part of Alexis Bénard's thesis (co-supervised with F. Vavre), we characterized the acute effect of stressors on the symbiotic association following the experimental design presented above (set up: part (a) at  $G_1$  on Figure 24, results: Figure 25).

As described in chapter 3.1, the presence of virus significantly reduced the larval and adult survival of the flies. On the other hand, *Wolbachia* had a significant protective effect on the survival of virus-infected adult flies (*via* food), which may be explained by a (non-significant) reduction in viral load in

*Wolbachia*-colonized individuals. On the bacterial side, the presence of DCV had no significant impact on *Wolbachia* density, nor on the composition of the bacterial population (in terms of Octomom copy number).

As presented in chapter 4.1.1, an acute stress of paraquat had no impact on life-history traits (emergence rate, survival), but reduced *Wolbachia* density. Using this set up, we confirmed these results and now show that this reduction did not impact bacterial composition (estimated by variation in Octomom copy number). No within-host selection was detected after one generation of stress, but the reduction in density could generate a bottleneck in bacterial transmission.

Let's now focus on the combined impact of oxidative stress and viral infection on the symbiotic association. As expected, the protective effect of *Wolbachia* was reduced in the presence of paraquat, a phenotype that can be explained by the reduction in bacterial density resulting from the paraquat treatment. This result confirms that bacterial density plays a major role in anti-viral protection. The median viral load did not appear to be affected 2 days after adult emergence, but its increased variance could lead to increased mortality of individuals with a high viral load later in life. In summary, the combination of stressors modifies the association response to viral stress in *Wolbachia*-infected flies.

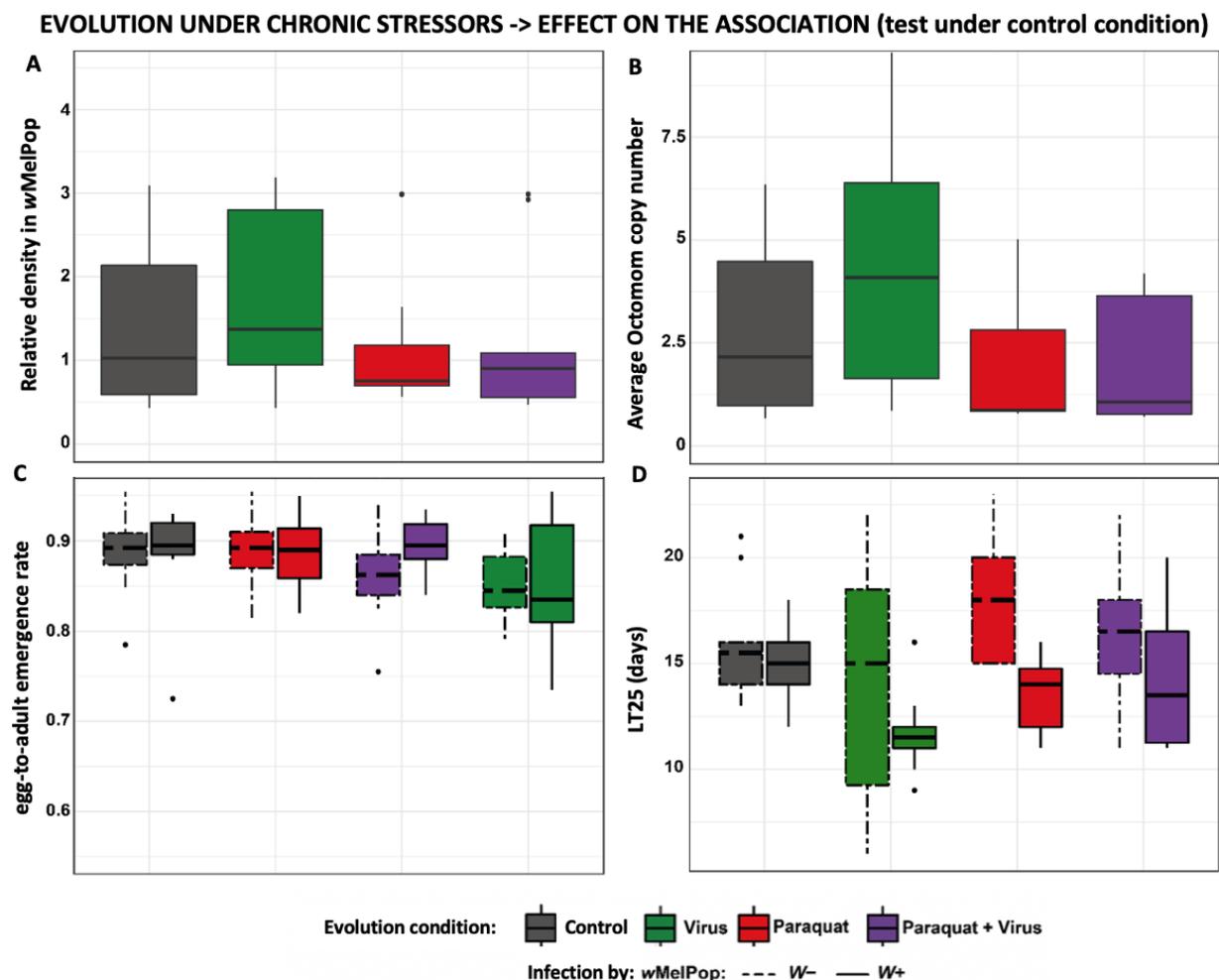


**Figure 25 | Influence of acute stressors on the symbiotic association.** **A.** Bacterial relative density, **B.** Average Octomom copy number; **C.** Viral load (log) 2 days after adult emergence; **D.** lethal time 25%. *Wolbachia* infection status is represented on the boxplot border (solid line = *Wolbachia*<sup>+</sup>; dotted line = *Wolbachia*<sup>-</sup>). Color within boxplots indicates stress condition (gray = control without stressor; green = DCV; red = paraquat; purple = combination of paraquat and DCV). Boxplot sharing the same letter are not significantly different (HDR post-hoc test,  $p > 0.05$ ).  $n = 10$  replicate lines / condition, 5-10 replicates/replicate line. Graphs from Alexis Bénard.

### 4.2.3 - Evolution under chronic stress

The results we obtained under acute stress condition indicate that the experimental conditions used can induce selective pressures on the different partners (paraquat: decrease in bacterial density, virus: decrease in fly survival; combination of stresses: loss of *Wolbachia*-mediated anti-viral protection). We therefore carried out an experiment to characterize the evolution of the symbiotic system in response to these different stressors (12 generations without stressor, with paraquat, with virus, or with paraquat and virus; 10 replicas/condition lines; experimental set up: parts (b, c) at G<sub>13</sub> on **Figure 24**). Indeed, we wanted to determine whether, in response to these experimental conditions, within- or between-host selection were detectable at the level of the bacterial population, and whether the host evolved by selection of resistance or tolerance mechanisms to the stressor.

- Under conditions of stress release (*i.e.*, test under control condition; part (b) at G<sub>13</sub> on **Figure 24**) after these 12 generations of evolution under stress, we did not detect any significant difference between evolution regimes (**Figure 26**) and/or between initial (G<sub>1</sub>, control condition, **Figure 25**) and final (G<sub>13</sub>, evolution condition, **Figure 26**) steps, either in bacterial population load/composition or in host survival.

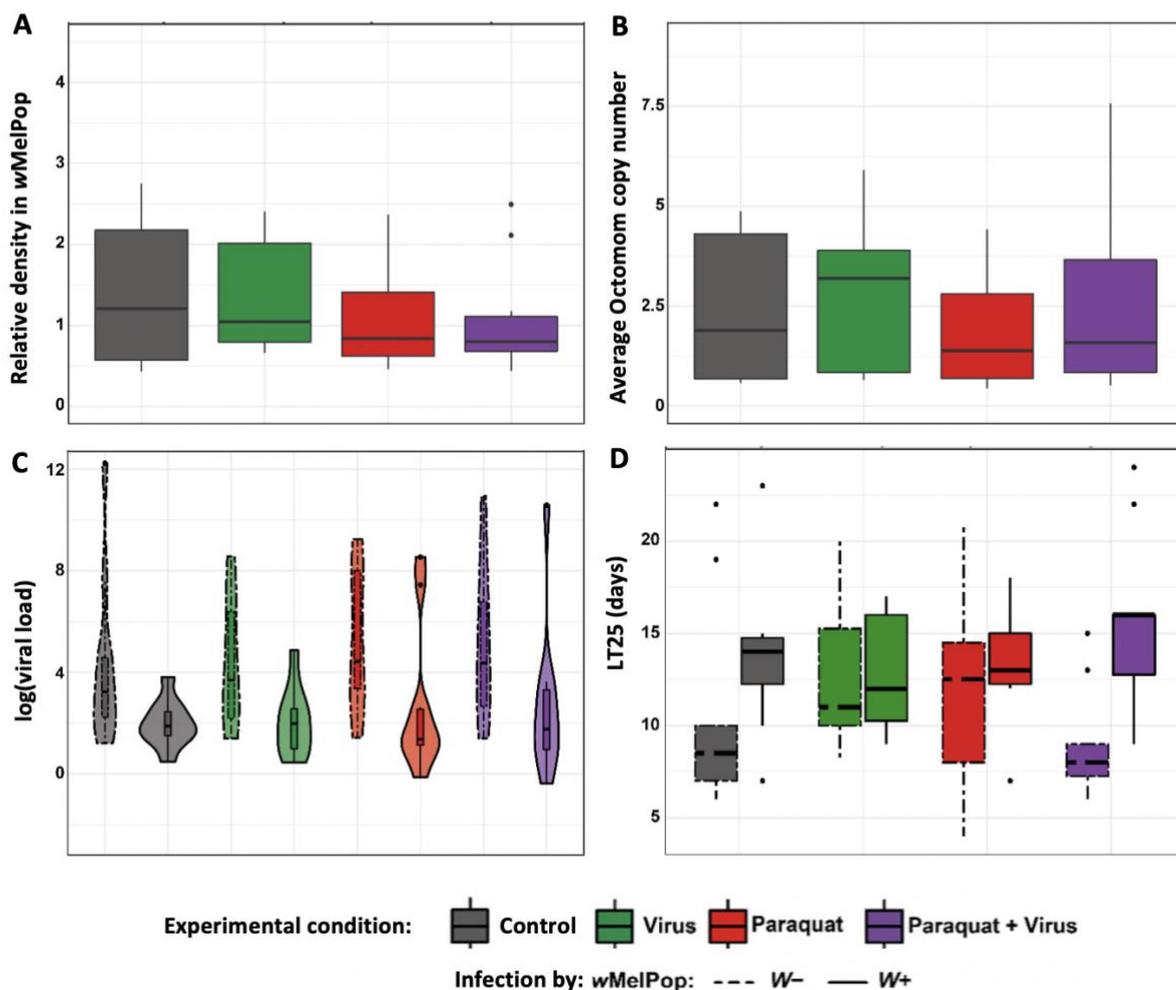


**Figure 26 | Influence of evolution under different stress regimes on the symbiotic association.**

All traits have been measured at G<sub>13</sub>, in absence of stressors. **A.** Bacterial density and **B.** Average Octomom copy number 2 days after emergence; **C.** Egg-to-adult emergence rate and **D.** Lethal time 25%. *Wolbachia* infection status is shown on the boxplot border (solid line = *Wolbachia*<sup>+</sup>; dotted line = *Wolbachia*<sup>-</sup>). Color within boxplots indicates stress condition (gray = control without stressor; green = DCV; red = paraquat; purple = combination of paraquat and DCV). n = 10 replicate lines / condition. 5-10 replicates/replicate line. Graphs from Alexis Bénard.

- Under conditions of viral stress (part (c) at G<sub>13</sub> on **Figure 24**), we expected an increase in antiviral resistance in aposymbiotic lines, linked to the selection of antioxidant mechanisms in lines that had evolved with paraquat, and of immune mechanisms in lines that had evolved with virus. We also expected an increase in antiviral resistance in symbiotic lines, linked to the absence of counter-selection of hyper-proliferative variants in paraquat-evolved lines and to the selection of hyper-proliferative variants or protective mechanisms in virus-evolved lines. However, we did not find any significant increase in bacterial density/composition, nor in antiviral resistance (**Figure 27**). Nevertheless, we found that evolution under viral and oxidative stress conditions had a non-significant tendency to increase the median survival of flies not colonized by wMelPop. The higher TL25 variance observed under these conditions could point to a partial tolerance to DCV in populations subjected to these evolutionary conditions.

### EVOLUTION UNDER CHRONIC STRESSORS -> EFFECT ON RESPONSE TO VIRAL INFECTION



**Figure 27 | Influence of evolution under different stress regimes on response to DCV infection.**

All traits have been measured at G<sub>13</sub>, after DCV ingestion during the larval stage. **A.** Bacterial density, **B.** Average Octomom copy number; **C.** Viral load (log) 2 days after adult emergence; **D.** lethal time 25%. *Wolbachia* infection status is shown on the boxplot border (solid line = *Wolbachia*<sup>+</sup>; dotted line = *Wolbachia*<sup>-</sup>). Color within boxplots indicates stress condition (gray = control without stressor; green = DCV; red = paraquat; purple = combination of paraquat and virus conditions). n = 10 replicas / condition. 5-10 replicates/replicate line. Graphs from Alexis Bénard.

Given the potential for response of the experimental system (both on the host and bacterial side), these results suggest that the adaptive potential of the association was limited, and that the drift taking place during symbiont transmission could play an important role in this limitation. These studies were very time-consuming, due to the large number of replicas/conditions to be tested and the variety of approaches used. Unfortunately, due to the covid crisis, it was not possible to maintain the lines in the evolutionary regimes for longer to test longer-term evolution.

#### **Related publications:**

Bénard A, Jacquet A, Guyot L, Henri H, Vavre F, Kremer N. Impact of oxidative stress and viral infection on the *Drosophila melanogaster* / *Wolbachia* symbiosis and its evolution. *In preparation*.

### 4.3 - Discussion

Oxidative stress is complicated to study. First, ROS are difficult to measure directly because of their short life. H<sub>2</sub>O<sub>2</sub> seems to be the more stable one, and can be stained *in situ* using probes (DAB, Amplex red, DCFH<sub>2</sub>...), but can be generated artefactually during sample preparation (Noctor *et al.* 2015). The effect of ROS on DNA damage, lipid peroxidation or protein carbonylation are more generally quantified, using technics such as comet assay, MDA assay kits or Oxyblots, respectively. However, these measurements must be balanced by the antioxidant capacity (*i.e.*, the amount of ROS that can be detoxified by all the antioxidants present in the organism; measured using an antioxidant assay kit) or the activity of individual detoxifying enzyme / buffering molecule (Superoxide dismutase, catalase, glutathione, carotenoids...). In addition, ROS are mediating signaling pathways and their localization can be a critical factor to determine cellular response. For all these reasons, we moved towards a multi-omics approach, that aims to examine the impact of oxidative stress on clusters of genes and metabolites, and to determine which respond together to oxidative stress. However, integrating all these levels of analysis is methodologically challenging (Sies *et al.* 2022). To make it more realistic, we studied the lifelong exposure to oxidative stress by performing a long-term exposure (whole larval development). In this context, we showed here that the impact of oxidative stress was not restricted to redox signaling, but more generally impacted metabolism at a whole, potentially impacting host physiology and life-history traits. Our experiment was also interesting in that it investigated the effect of a combination of stress factors, showing that one stress factor can modulate the effect of a second. Combinations of stress factors are common in nature, particularly as a result of anthropization and global warming, but they are not often taken into account in scientific studies.

In order to study rapid adaptation of a symbiotic system to environmental changes, we chose to perform experimental evolution. Indeed, letting a partner evolve under controlled conditions appears to be key approach to determine how partners evolve. If we focus on evolutionary response to DCV pricking, other experimental evolution experiments showed that mutations either on fly genes (viral restriction factor *pastrel*; Martinez *et al.*, 2016) or *Wolbachia* genes (conferring a higher load; Faria *et al.*, 2016) were selected for after a dozen of generations and increased resistance to DCV injection. Because the infection by natural feeding exerted a selective pressure at the adult age at which we allowed flies to lay eggs for the next generation, we expected that our experimental evolution protocol would select for flies that survive better after DCV infection. This could include the selection of bacterial genes which encode for a higher replication rate or density (*e.g.*, an increase in the number of Octomom copies), and/or the selection of DCV resistance alleles others than *pastrel*. In our experiment however, we did not detect any effect of the evolution regime on the antiviral protection,

nor on the evolution of the bacterial density or composition. As the *Octomom* copy number is heritable and can be (at least artificially) selected (Chrostek & Teixeira 2015), these results could suggest that the selective pressure was not strong enough. Alternatively, the benefit of harboring protective symbionts could be counter-balanced with the cost of their presence within host tissues (Chrostek & Teixeira 2015) and would thus suggest that *Wolbachia* density (and indirectly the protective effect) was already at its optimal level. Finally, the most likely hypothesis is that efficiency of selection was reduced because of drift on the symbiont population during transmission and/or on the host population (Bénard *et al.* 2021). To conclude, symbiosis is often considered as a source of adaptation for hosts to new environments (Hussa & Goodrich-Blair 2013; Sudakaran *et al.* 2017), as the acquisition of new symbionts can constitute a rapid response on a host population scale, when biotic / abiotic constraints are stable while imposing strong selection pressure, and when the benefits provided by the symbionts outweigh their costs. However, once these associations are already established, symbionts may have little adaptive potential, due to reduced genetic diversity and the strong influence of genetic drift.

More generally, understanding rapid adaptation or studying the influence of ecological strategies (*e.g.*, specialist vs. generalist symbionts) on the evolution of symbiotic ecosystems requires to take into account the mechanisms of plasticity and selection. These questions remain largely unanswered, due to the complexity of characterizing eco-evolutionary dynamics within and between hosts, the difficulty of identifying markers of plasticity and the impact of different levels of selection within the host ecosystem (and between host ecosystems). The development of methods for measuring fitness traits at different scales, and of multi-scale models of eco-evolution or adaptive dynamics including several partners within a host, would enable these questions to be addressed more widely.

## Discussion and perspectives

As I introduced before, a new paradigm now tends to consider the host as an holobiont, or even an ecosystem. The holobiont refers to a host and its community of associated microorganisms as an 'integrated' unit. Recently, it has been proposed that holobiont is the primary level of selection in evolution (Rosenberg & Zilber-Rosenberg 2016). However, in order for the selection to act, three main criteria must be taken into account (Hurst 2017):

- First of all, inter-individual variation should be present in the symbiotic community. This is the case in the symbiotic systems I studied, as different populations of *Vibrio fischeri* have been identified (Wollenberg & Ruby 2009; Bongrand *et al.* 2016), gut microbiota of fruit flies is highly diverse, and variation within the wMelpop strain is present, at least at the level of the Octomom region.
- Second, the variation observed in the symbiotic community should be associated with variation in host fitness. In *V. fischeri*, the strains can have a different colonization behavior and infection dynamics (Bongrand *et al.* 2016; Bongrand & Ruby 2019). However, these studies relate to competition between strains and adaptation to squid tissues, and do not directly address the fitness benefit for the squid to harbor one of these variants. As the benefit of harboring bioluminescent bacteria is mostly an ecological benefit (to avoid predation), this is complicated to test it experimentally. In fruit flies, some bacterial species (such as *Lactobacillus* and *Acetobacter*) are known to have a beneficial impact on larval growth, but the fitness effect of the majority of the gut microbial species is not well characterized (and is *a priori* mostly neutral). As for *Wolbachia*, the correlation between bacterial density and anti-viral protection is well established in our studies and others, but can also be linked to fitness costs in absence of virus (Chrostek *et al.* 2013; Chrostek & Teixeira 2015; Martinez *et al.* 2015; Monnin *et al.* 2021).
- Third, symbionts having a fitness effect must be directly or indirectly heritable from parents to offspring. In vertically transmitted symbioses, such as the *Drosophila-Wolbachia* symbiosis, the direct heritability through reproduction ensures a strong correlation of interests between the host and the symbionts, suggesting that the holobiont can be a selection level. However, it does not mean that other levels of selection cannot act, as competition between variants (within-host and between-host competition) can create conflicts and nucleo-cytoplasmic conflict can also occur. In the gut microbiota of larval fruit flies, the host-associated symbiotic community is diverse and partner fidelity is weak, limiting the 'alignment of interests' between host and symbionts, and the relevance of considering the holobiont as a primary selection level. In the squid-vibrio system, even we face horizontal transmission through the environment, the fidelity of partners is strong (because of selective processes and co-development (Nyholm & McFall-Ngai 2021)), and the importance of the holobiont as a level of selection is more clear than in other horizontally-transmitted symbioses.

Overall, the three models of symbiosis I have studied confirm the fact that considering the holobiont as a primary level of selection is not always relevant (Moran & Sloan 2015; Hurst 2017), and that all symbioses (in the context of holobiont) are not necessary adaptive. To better understand the diversity and evolution of holobionts (from now on limited again to the definition of hosts and their symbionts), we should thus rather consider them as general ecosystems.

An ecosystem, from the Ancient Greek οἶκος for "house" and σύστημα for "organized body", represents an ecological community consisting of different populations of organisms that live together in a particular habitat and face various environmental constraints. Considering hosts as ecosystems opens the way we study them by integrating the role of the environment in shaping microbiota composition (*e.g.*, influence of diet or environmental stressors), the questions of community assembly

(*e.g.*, keystone species, succession of species) and the ecological dynamics of microbial communities (*e.g.*, synergy, competition, replacement). It also allows to take into account the influence of within-host evolution that is often neglected. In addition, ecosystems are dynamic, so we should consider holobionts as a system which can exhibit rapid dynamics, even on the scale of a diel cycle, and study evolutionary trajectories. Interestingly, host are not homogeneous environments, and each tissue within the host has some specificities in terms of cellular organization, gene regulation, connection with other cells/tissues, selective pressures... They form various ecological niches that can be considered as 'environmental' constraints by symbionts, especially endosymbionts such as *Wolbachia*, which have an intracellular lifestyle (Sicard *et al.* 2014). Studying molecular interactions between host and symbionts (but also between symbionts) in these different niches should help to understand the mechanisms involved (shared or specific) but also the selective pressures underlying these interactions. While I have tried to start integrating some of these concepts/methods into my research projects, there is still a long way to go (and one that needs collaborations and networking to succeed!).

Although I am not specialist in philosophy of science, I would like to come back briefly to the question of immunity and individuality. As discussed by Thomas Pradeu (Pradeu 2019), the concept of immunological individuality is intrinsically linked to that of self and non-self. Without going into details, this concept is switching from a definition based on the ability to trigger an immune response to a framework which considers an organism as a dynamic ecosystem in interaction with its environment (Pradeu 2019). This new framework is in line with what I developed in this manuscript, and highlights that time (*i.e.*, infection dynamics, coevolution) and space (*i.e.*, tissue specificity) seem to be very important factors to consider.

But many questions are open, such as where are the limits of self and environment? How should we consider protective symbionts in the context of immunity, as these entities are micro-organisms (tolerated -to a certain degree- by the host) and defend at the same time the host against pathogenic microorganisms? How should we envision stressors such as oxidative stress, which are both environmental changes and central effectors of many immune or developmental signaling pathways? How should we think about metabolites that modulate host vigor and competition between micro-organisms? More generally, should we redefine immunity in a broader sense that includes not only canonical immune pathways but also protective symbionts, reactive oxygen species and metabolism?

I will conclude this section by saying that studying symbiosis is a huge and fascinating field of research, especially now that its impact is increasingly recognized as a major force in biology, ecology and evolution (McFall-Ngai *et al.* 2013). What I like, and would like to continue developing, is the need to study it in an integrative way, at different scales (cell, tissue, individual, population) and with different approaches (microscopy, molecular biology, biochemistry, ecology, evolution). Considering symbiosis from an ecosystem point of view also enables us to address more general issues relating to the environment (*e.g.*, climate change), health (*e.g.*, the emergence of infectious diseases), and more generally to promote the concept of "One health".

In the next part, I will present my most recent research projects, which ingrate the question of symbiosis in a more applied way. In just a few pages, I will also outline my implications for the environment and society in general.

# CURRENT PROJECTS & PERSPECTIVES

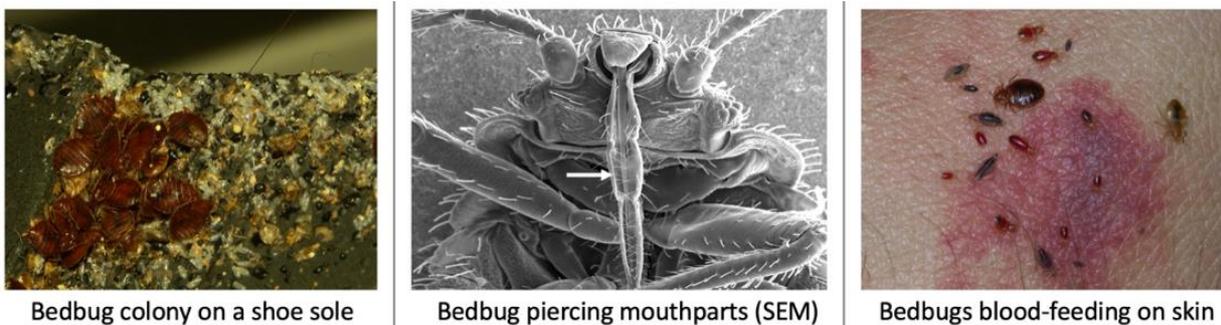


# Chapter 1 – Symbiosis in bedbugs

## 1.1 - Bedbugs: a new study model in the context of biological control

Bedbugs (*Cimex lectularius*, Hemiptera; Cimicidae; **Figure 28**) feed solely on blood during the night, causing severe reactions to bites, and in some cases, urticaria, anaphylactic reactions or permanent scarring (Doggett *et al.* 2012). The psychological impact of bedbugs is also considered as potentially severe (anxiety, paranoia, sleep disorder), with symptoms persisting long after the elimination of the infestation (Doggett *et al.* 2012).

Bedbugs have proliferated since the 19<sup>th</sup> century following urbanization and global trade, but the massive use of insecticides in the mid-20<sup>th</sup> century has suddenly stopped this progression. Nevertheless, a resurgence of bedbug infestations is observed worldwide since the 1990s (Davies *et al.* 2012), and France is not an exception. This resurgence can be explained by various factors, first and foremost the evolution of resistance to insecticides (in particular to pyrethroids, which are widely used), but also globalization and second-hand market. Despite their limited capacities of active dispersal, bedbugs thus colonize new places extremely rapidly due to human movements, and become major health and economic problems (Scarpino & Althouse 2019).



**Figure 28 | Bedbugs**

Sources: Pictures 1 and 3: [https://www.flickr.com/photos/lou\\_bugs\\_pix/](https://www.flickr.com/photos/lou_bugs_pix/);

Picture 2: <http://www.freestockphotos.biz/stockphoto/16710>

Blood is a nutritionally unbalanced diet, lacking in B vitamins in particular. Like many other hematophagous insects, bed bugs host a bacterial endosymbiont that complements their diet, here a strain of bacteria belonging to the *Wolbachia* genus (Hosokawa *et al.* 2010). Indeed, *wCle* provides riboflavin and biotin necessary for the fertility (egg production and hatching) and nymphal development of bedbugs (Hosokawa *et al.* 2010; Moriyama *et al.* 2015; Hickin *et al.* 2022). While the riboflavin pathway is present in the common ancestor of *Wolbachia*, the biotin pathway was acquired more recently by *wCle* through horizontal transfer from symbionts infecting hematophagous hosts (Nikoh *et al.* 2014).

Bedbugs can also harbor two facultative endosymbionts: a Torix group *Rickettsia*, which infests 60% of lab populations and impacts very slightly nymphal development and fertility (Thongprem *et al.* 2020); and a BEV<sup>15</sup>-like  $\gamma$ -proteobacterium, now classified as a symbiopectobacterium (Nadal-Jimenez *et al.* 2022), whose phenotypic impact on bedbug physiology/fitness is currently unknown, but which seems to be highly prevalent in natural populations (Hosokawa *et al.* 2010; Meriweather *et al.* 2013). All three

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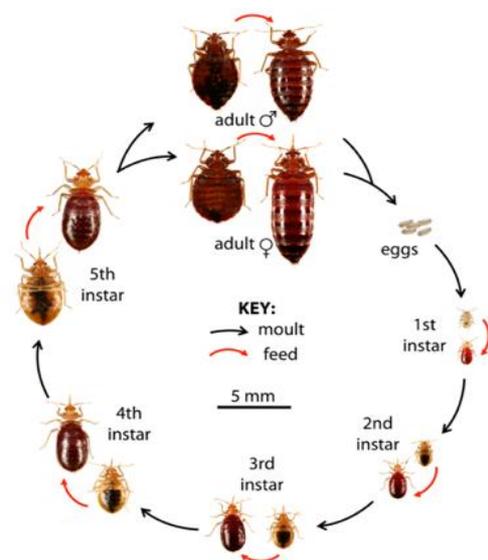
<sup>15</sup> BEV : Bacterium of *Euscelidius variegatus* (leafhopper) (Degnan *et al.* 2011)

endosymbionts are mainly located in a specialized organ, the bacteriome, but also in the insect ovaries, which ensure their maternal transmission (Thongprem *et al.* 2020).

Because of the urgent need to limit bedbug infestations, discussions between a company specialized in the fight against invasive species (Romain Lasseur, IZInnovation Lyon), researchers at the LBBE (Fabrice Vavre, Julien Varaldi) and at the BF2i (Aziz Heddi, Anna Zaidman-Rémy), and the opportunity to use the Symbiotron<sup>16</sup>, we decided to establish a new facility to study bedbugs. On the one side, it is crucial to better characterize the diversity, the spatial distribution and the relative contribution of the genetic determinants underlying resistance (*e.g.*, target-site insensitivity, insecticide detoxification, cuticle thickening (Dang *et al.* 2017; Balvin & Booth 2018)). On the other side, there is a need to develop alternative and complementary methods to by-pass the use of insecticides in the mid/long term. Using the rupture of the obligate nutritional symbiosis appears to be a promising way to develop specific control methods. In this context, I obtained a grant from the Region Rhône-Alpes (SymBed) and another one from the Agence Nationale de la Recherche (FBI) to study these questions. The part relative to the characterization of pyrethrinoid resistance (developed by Julien Varaldi, Fabrice Vavre, Romain Lasseur and Chloé Haberkorn) will not be developed here. I will rather focus on the questions and projects relative to the interaction between bedbugs and their symbionts, which fits to the expertise I gained with my previous research work. In this context, I co-supervised two PhD students together with Anna Zaidman-Rémy (BF2i) and Fabrice Vavre (LBBE), funded through the Symbed project: Marius Poulain (CIFRE contract with IZInnovation, 2020-2023), and Raphaël Jorge (Region Rhône-Alpes, 2022-2025).

## 1.2 - Study of the interactions between bedbugs and their symbionts

### 1.2.1 - Bedbugs: life-history and rearing



**Figure 29 | The life cycle of bedbugs**

Source:

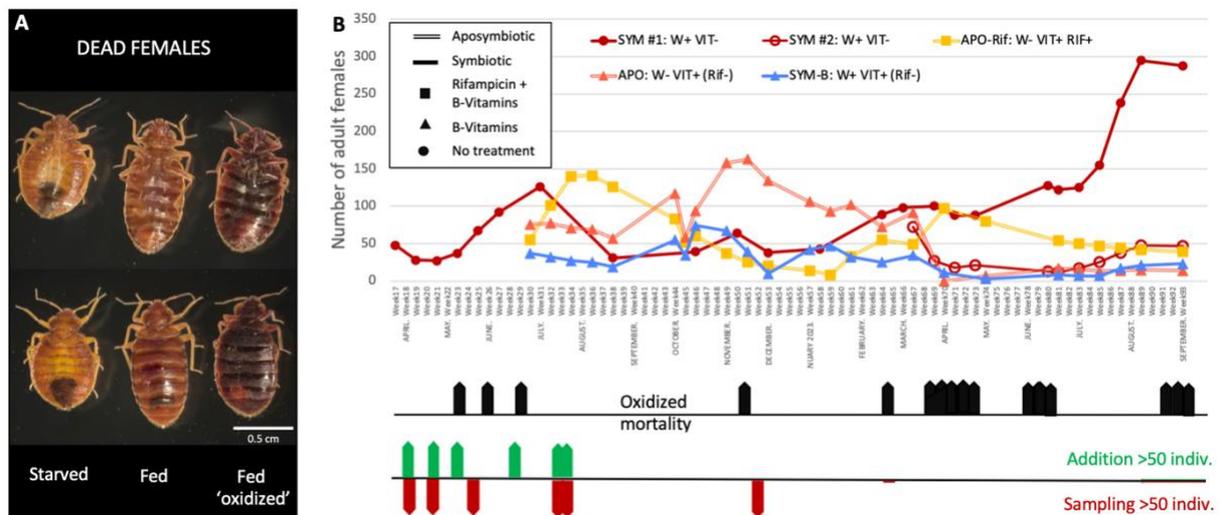
<https://www.bedbugfoundation.org/>

Bedbugs are strict hematophagous insects, which feed before each nymphal transition and regularly (males and females) at the adult stage (Figure 29). We thus set up at the Symbiotron a rearing system using an artificial feeding system (Hemotek), and partnered with the Etablissement Français du Sang (EFS) for the use of human blood to mimic, as possible, physiological conditions. Based on the literature, we selected the following rearing conditions (climate chambers): 24°C, 60% RH and LD 12:12.

Surprisingly -as bedbugs are expanding rapidly- it remains very difficult for us to raise them in artificial breeding. Oliver Otti, from the University of Dresden, was very helpful in supplying us with bedbugs, giving us many tips on bedbug ecology and optimizing our protocols. Nevertheless, feeding bedbugs on artificial feeding systems seems to limit the expansion of colonies, even though we used human blood. And more importantly,

<sup>16</sup> a new structure from the FR BioEEnViS, dedicated to the study of insect/pathogen interactions in confined environments

chronic events of mortality regularly occur, accompanied by an ‘oxidized phenotype’ in which blood content from the gut permeates the whole body, oxidizes, and turns the bedbugs dark brown. The characterization of this phenotype will be described below, but overall, these mortality events strongly limit our ability to perform experiments (Figure 30, oxidized mortality peaks in black).



**Figure 30 | Difficulties encountered in increasing the size of bedbug populations**

**A.** Ventral side (top) and dorsal side (bottom) of bedbugs starved, fed or fed and exhibiting an ‘oxidized phenotype’. **B.** Evolution of the breeding size of different populations over time. Black arrows below the graph symbolize oxidized mortality peaks (more than 40% loss of insects in a population), green arrows represent events in which more than 50 adult females were added to the SYM#1 population, and red arrows represent sampling events in which more than 50 adult females were removed from the SYM#1 population.

Pictures/graphs: Chloé Haberkorn (A) and Elodie Rosinski (B).

### 1.2.2 - Control of symbiotic communities

When pests are dependent on symbiotic bacteria for a supply of nutrients, it is possible to develop methods to eliminate or reduce the symbiotic load, in order to reduce insect performance. These methods can be applied to numerous insect pests in agriculture (*e.g.*, phytophagous hemipterans, certain xylophagous and beetle pests of grain stocks) or health (*e.g.*, tsetse flies, lice, but also bedbugs). However, the elimination of symbionts by an antibiotic treatment is hampered by the need to reduce antibiotic spraying, both to limit the loss of specificity of the control method and the evolution of antibiotic resistance. To target the mechanisms of dependence and disruption of the association, it is therefore necessary to understand the mechanisms of interaction between the host and its obligate symbiotic bacterium. For example, the control of the symbiotic bacterium *Sodalis pierantonius* by the weevil *Sitophilus oryzae* is linked to the secretion by the host of an antimicrobial peptide, coleopteracin A (Login *et al.* 2011), and the elimination mechanisms of the obligate symbiont *Buchnera aphidicola* by the pea aphid *Acyrtosiphon pisum* are linked to the induction of the lysosomal pathway and the Rab7 protein (Simonet *et al.* 2018).

But before exploring the mechanisms involved in bacterial regulation in bedbugs, it is important to characterize the dynamics of symbionts during the insect life cycle. A previous study has shown that *Wolbachia* relative density increases between the first and the 5<sup>th</sup> nymphal instar, and that density decreases in adult upon starvation (Fisher *et al.* 2018). However, this study did not explore variations between nymphal stages, nor the influence of sex or ageing in adults (independently of starvation). In the PhD project of Marius Poulain, we thus decided to get a better definition of the dynamics of

infection. The use of cohorts <sup>17</sup> limited the number of samples we could obtain, but we eventually got 6 cohorts to study the evolution of bacterial density during nymphal development according to feeding, and 6 other cohorts to study the influence of sex, ageing and starvation in adults. We initially quantified by duplex qPCR the relative density of *Wolbachia* in all these samples (protocols from Fisher *et al.* 2018, Marius Poulain & H el ene Henri). We completed this dynamic using Fluorescent *In Situ* Hybridization (FISH): we localized *Wolbachia* in whole insects and tissues (bacteriome and ovarioles/ovaries) through development and characterized the evolution of bacteriome size (Marius Poulain & Severine Balmand). Because the dynamics of BEV-like density has not been described yet, we took advantage of this sampling effort and developed a duplex qPCR to quantify the relative density of BEV-like in the same samples (Rapha el Jorge & H el ene Henri).

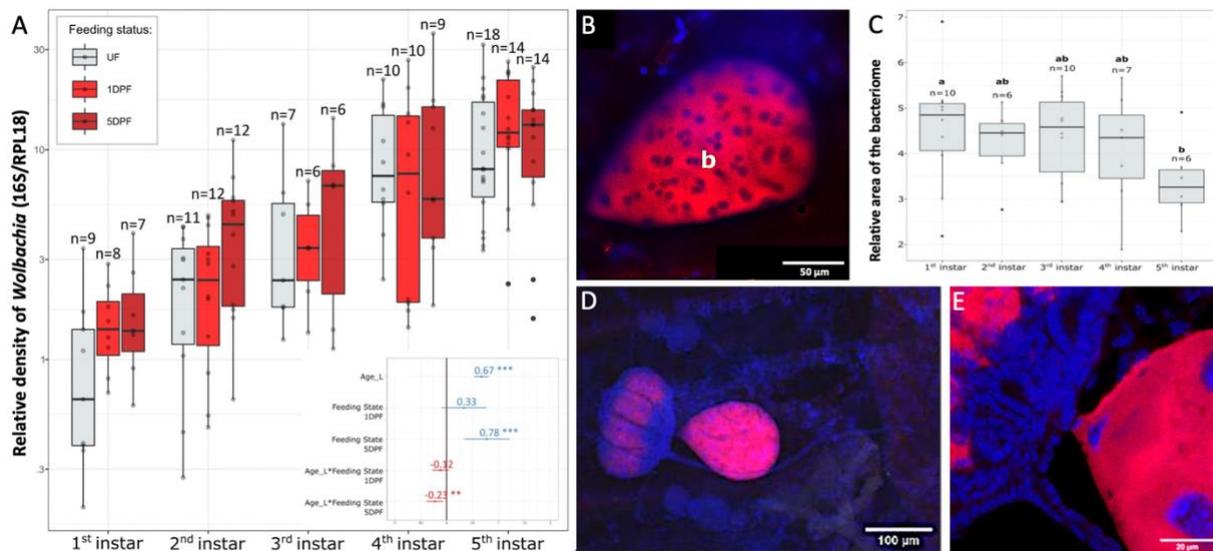
- *Wolbachia*:

Concerning the dynamics of *Wolbachia*, we confirmed that the relative density increases over nymphal development (Fisher *et al.* 2018), and we showed that this increase is exponential between the 1<sup>st</sup> and the 5<sup>th</sup> nymphal instar. In addition, it appears that overall, *Wolbachia* density was higher in individuals 5 days post-feeding compared to unfed individuals (**Figure 31A**). This result could either be due to blood feeding or to development, but it was not possible to tease apart these two effects in this experiment, as a blood feeding is required for molting and continuation of the cohorts. Complementary experiments comparing fed and unfed nymphs at a specific stage could inform about the direct effect of blood feeding on this dynamic. In the bacteriome (**Figure 31B**), the increase in relative density was due to faster multiplication of the bacterial cells than the host cells, suggesting an intense proliferation of *Wolbachia* within cells during development. These results are reinforced by the decrease of the relative size of the bacteriome over development (**Figure 31C**). Interestingly, we showed evidence of bacterial colonization in bacteriomes and ovaries as early as the first nymphal stage. The developmental origin of bacteriomes is not clearly defined in bedbugs, but the physical connection between bacteriomes and ovaries up to stage 5 (**Figure 31D,E**) suggests a common tissue origin, and potentially an initial colonization as early as embryonic primordials.

In adults (**Figure 32A-D**), we showed that *Wolbachia* relative density was higher in females than in males, which could be explained by the presence of *Wolbachia* in ovaries and developing eggs. Further experiments aimed at specifically quantifying the relative density of *Wolbachia* separately in ovaries and bacteriomes are planned to determine if *Wolbachia* dynamics are similar in both tissues. Interestingly, even when bedbugs were regularly fed, relative *Wolbachia* density decreased in adult females during the 4 first weeks. This decrease in density could be due to a change in bacterial regulation (active through immunity or digestion, or passive through a decrease in available nutrients) or to a loss of *Wolbachia* via egg laying. In males however (**Figure 32D**), feeding induced an increase in relative bacterial density, suggesting that the bacterial population in the bacteriome continues to proliferate when blood intake persists.

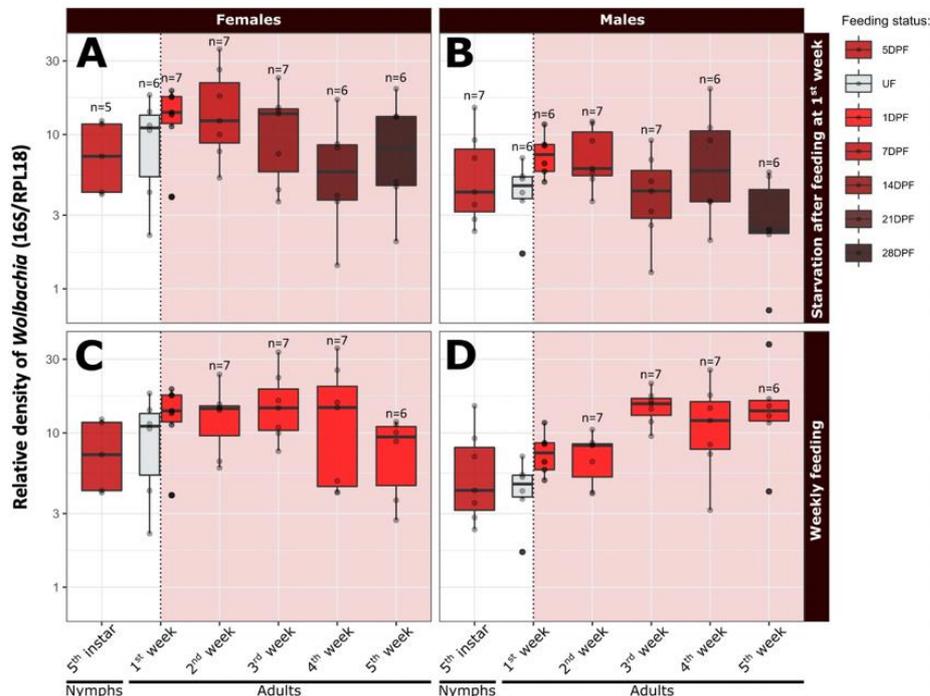
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<sup>17</sup> The protocol of **cohort** consists of letting a group of females lay eggs within a one-week window, and allowing the eggs develop to the target stage, while sampling at each stage and discarding individuals that have not molted within the 'canonical' timeframe (1 week). This leads to synchronized individuals from the same pool of females.



**Figure 31 | Dynamics of *Wolbachia* over development.**

**A.** Dynamics of the relative *Wolbachia* density (*i.e.*, *w*Cle 16S / RPL18; log scale) over 5 nymphal stages, according to the feeding status (UF: unfed, 1DPF: 1 day post feeding; 5DPF: 5 days post feeding). The box on the bottom right-hand corner indicates the summary of the LMER analysis on log-transformed data, showing a positive effect of development (stronger effect in UF compared to 5DPF), as well as a positive effect of feeding state (5DPF>UF). **B.** FISH image of a 4<sup>th</sup>-instar bacteriome (b) with *Wolbachia* in red and polylobed nuclei in blue. **C.** Evolution of the relative area of the bacteriome (*i.e.*, bacteriome area / thorax area) over 5 nymphal stages. Boxplot sharing the same letter are not significantly different (t-test,  $p > 0.05$ ). **D.** FISH image of an infected ovary together with its associated bacteriome in a 4<sup>th</sup>-instar nymph. **E.** Zoom of the connection between an ovary and a bacteriome. (Pictures and graphs: Marius Poulain).

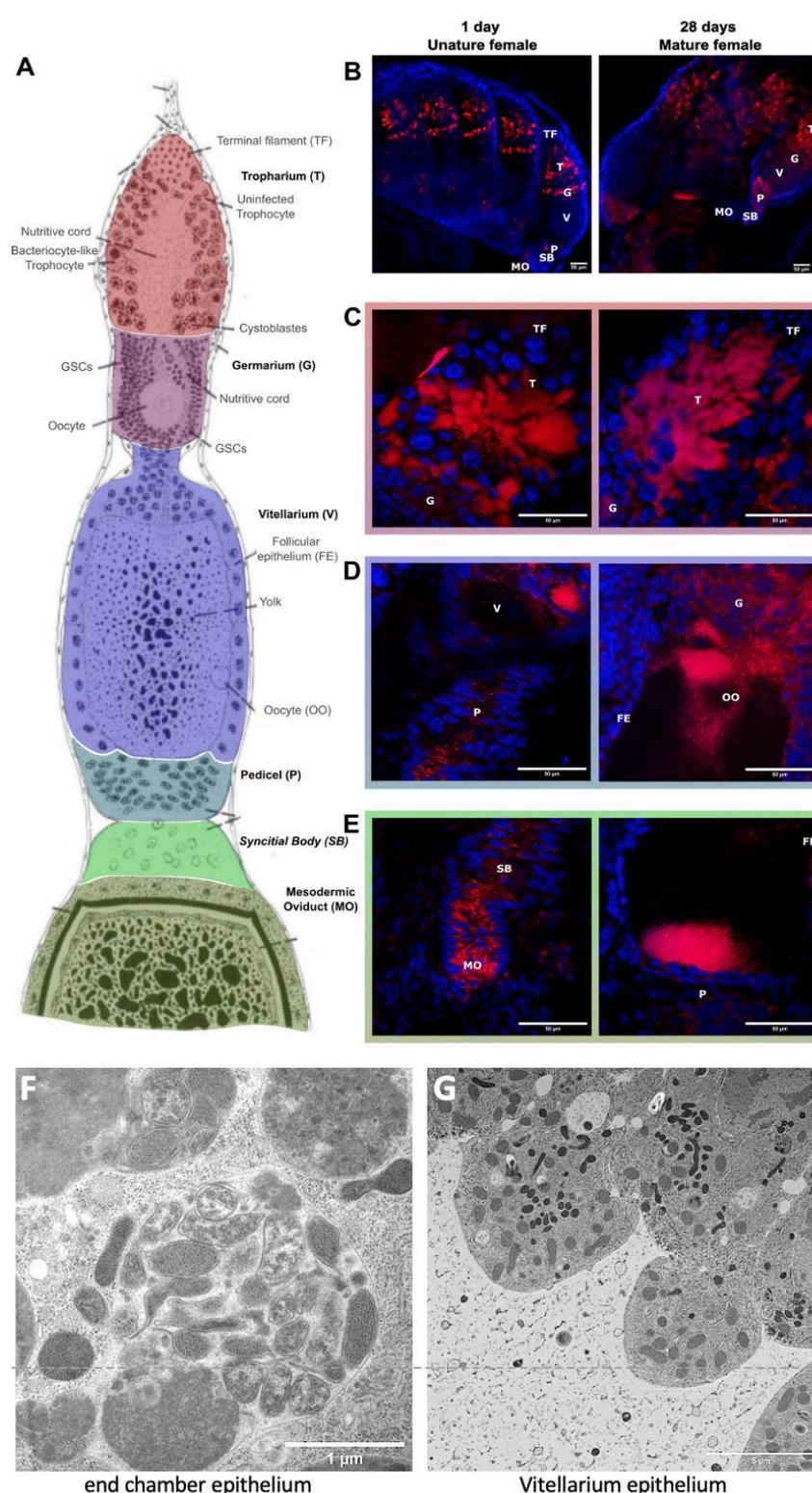


**Figure 32 | Dynamics of *Wolbachia* at the adult stage.**

**A-D.** Dynamics of the relative *Wolbachia* density (*i.e.*, *w*Cle 16S / RPL18; log scale) in adults, according to sex, ageing and feeding status (UF: unfed, DPF: day post feeding; all individuals have been fed at week 1, but half of the cohorts were starved until 28DPF (panels A & B), while the other half remained fed every week (panels C & D)). Graphs: M. Poulain).

We also wanted to describe more precisely the presence of *Wolbachia* within cells of the ovarioles and thus performed FISH and Transmission Electron Microscopy (TEM) with cryofixation, in collaboration with Severine Balmand (BF2i) and the CT $\mu$  (FR BioEEnviS). *Wolbachia* localization varied according to the different regions of the ovariole, and tended to colonize the vitellarium in concert with ovariole

maturation/oogenesis (Figure 33A-E). Interestingly, we could observe signs of *Wolbachia* digestion within phagosomes in the epithelium of the egg chambers of some samples (Figure 33F). Complementary TEM observation and lysosomal/autophagic staining are foreseen, to determine if this process is common, stage-specific, and leads to a regulation of *Wolbachia* density. We also observed many *Wolbachia* cells in the epithelium of the vitellarium facing the internal lumen, in cell apical membrane outgrowths (Figure 33G). Whether *Wolbachia* cells plays a role in the transmission or the provision of nutrients remains to be determined.



**Figure 33 | Localization of *Wolbachia* in the ovaries.**

**A.** The companion image shows the structure of the ovariole (Davis 1956). The color code indicates the following regions of the ovariole: tropharium (T, red), germarium (G, purple), vitellarium (V, blue), pedicel (P, cyan), syncytial body (SB, green), and mesodermic oviduct (MO, khaki). The same abbreviations are used in all the panels. **B-E.** FISH images of an adult ovary at 1 (unmature ovary) or 28 days (mature ovary); blue: nuclei (DAPI); red: *Wolbachia*. **F.** TEM image of a 'digestion body' where *Wolbachia* cells are getting degraded **G.** TEM image of the cells containing numerous *Wolbachia* facing the lumen of the vitellarium. (Pictures and graphs: Marius Poulain).

## Related publications:

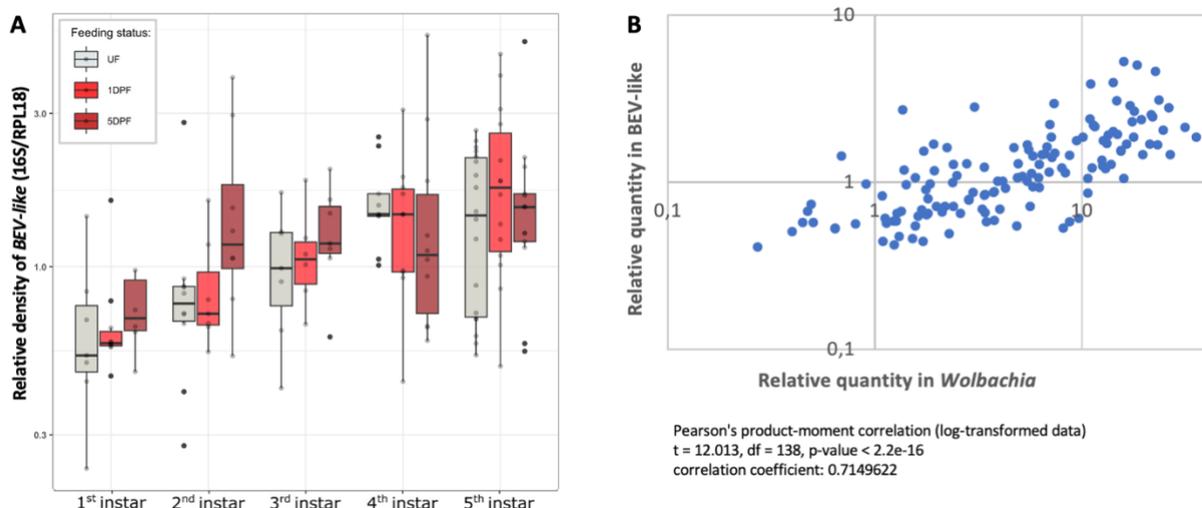
Poulain M, Rosinski E, Henri H, Balmand S, Delignette-Muller ML, Heddi A, Lasseur R, Vavre F, Zaidman-Rémy A, Kremer N. Development, feeding and sex shape the relative quantity of *Wolbachia*, the nutritional obligatory symbiont of bed bugs. Submitted to *Frontiers in Ecology and Evolution*, special issue on *Wolbachia*.

Poulain M, Balmand S, Rosinski E, Heddi A, Lasseur R, Vavre F, Zaidman-Rémy A\*, Kremer N\*. Infection dynamics of *Wolbachia* in bedbug ovaries. *In preparation*.

- Addendum to the initial project: BEV-like

*Wolbachia* is fixed in *Cimex lectularius*, but another symbiont, BEV-like, seems to be also present at high prevalence. In addition to be located in the ovaries and bacteriomes, BEV-like is also located in the Malpighian tubules (Hosokawa *et al.* 2010), and could play a role in the excretory system. In its native host (the leafhoppers *Euscelidius variegatus*), BEV is also present in the epithelium of the midgut and is pathogenic, reducing both longevity and fecundity (Purcell & Suslow K.G 1987; Cheung & Purcell 1993). The phenotype observed in case of pathogenesis is an atrophied digestive system, a reduction of the midgut glycocalyx, and a deteriorated cell membrane epithelium (Cheung & Purcell 1993). Because of its high prevalence in bedbug populations and its potential role in physiology and/or pathology, we decided to study the dynamics and potential fitness effects of this symbiont in bedbugs.

We first used samples collected by Marius Poulain over bedbug development, to determine BEV-like density in each nymphal stage and the potential impact of feeding on its density (Figure 34). As for *Wolbachia*, we detected an exponential increase of the density from the first to the 5<sup>th</sup> nymphal instar (significant increase by 1.7 at each stage). Both relative densities were correlated, which suggests a common regulation mechanism for both symbionts, but the increase over development was stronger for *Wolbachia* than for BEV-like. We noticed that the variance between samples with an experimental condition was high (and stronger than for *Wolbachia*), which could partially explain the smaller positive effect of blood feeding.



**Figure 34 | Dynamics of BEV-like over development.**

**A.** Dynamics of the relative BEV-like density (*i.e.*, BEV-like 16S / RPL18 by qPCR; log scale) over 5 nymphal stages, according to the feeding status (UF: unfed, 1DPF: 1 day post feeding; 5DPF: 5 days post feeding). LMER analysis on log-transformed data show a positive effect of development. The effect of feeding state (5DPF>UF) is highly variable according to stage. **B.** Positive correlation between BEV-like and *Wolbachia* relative densities.

These data will be supplemented soon by an analysis at the adult stage, to determine whether BEV-like dynamics follows that of *Wolbachia*, particularly with regard to starvation and sex. To determine any potential spatial competition between *Wolbachia* and BEV-like in bacteriomes, ovaries and Malpighian tubules, the co-localization of symbionts will be assessed by FISH.

- Mechanisms of symbiont regulation

To be able to determine the influence of *Wolbachia* ( $\pm$  BEV-like) on insect fitness and gene expression, we need to produce aposymbiotic individuals, in which *Wolbachia* ( $\pm$  BEV-like) has been eliminated. We thus started to set up a protocol (curing with Rifampicin) **to obtain aposymbiotic bedbugs and to establish a stable aposymbiotic line**, using complementation with a cocktail of B-vitamins (Hickin *et al.* 2022).

To confirm the dependence phenotype (Hosokawa *et al.* 2010; Hickin *et al.* 2022) in our lines, we compared the number of eggs laid by adult females (and the corresponding hatching rate) in different rearing conditions:  $W^+Vit^-$ ,  $W^+Vit^+$ ,  $W^-Vit^+$ , and  $W^-Vit^-$  (where B-vitamin complementation stopped either at the 5<sup>th</sup> nymphal stage or at the adult stage). We confirmed that the number of eggs laid significantly decreased in the  $W^-Vit^-$  condition. Interestingly, it slightly decreased when B-vitamin complementation was stopped at the adult stage, while it almost dropped to zero when the B-vitamin complementation was stopped at the 5<sup>th</sup> stage. Because bedbugs hatch with immature oocytes, it suggests that B-vitamins are mostly required for the development of egg chambers or the production of germ cells, and in a lesser extend for the maturation of oocytes from germ cells after hatching. Surprisingly, we did not manage to restore the symbiotic phenotype in  $W^-Vit^+$  in this control experiment, although we were able to create a stable  $W^-Vit^+$  line in our rearing (that we checked several times for the absence of *Wolbachia*). However, in the control experiment, bedbugs were reared in small effective sizes, which strongly delayed the development time and seems to be suboptimal for bedbug development.

As we do not have bedbug populations lacking BEV-like in our rearing facility (as in other European labs rearing bedbugs), we first need to determine if the antibiotic treatment eliminates only *Wolbachia* or both symbionts, in order to best interpret our results. Hosokawa *et al.* (2010) highlighted the dependence phenotype in individuals lacking BEV-like bacteria. However, it does not mean that BEV-like does not participate to the provision of B-vitamins...

**To identify host genes putatively involved in the control and the maintenance of the symbiotic population**, we also plan to compare the RNAseq reads (Illumina sequencing) isolated from symbiotic and aposymbiotic insects. First, we plan to determine the influence of *Wolbachia* ( $\pm$  BEV-like) and/or B-vitamins on host gene expression, particularly in terms of immune control (in the bacteriome) and reproduction (in the ovaries, 48 h after female hatching). We will thus compare gene expression in bacteriomes and in ovaries between the  $W^+Vit^-$ ,  $W^+Vit^+$  and  $W^-Vit^+$  rearing conditions<sup>18</sup>. Dissection of aposymbiotic bacteriomes is tricky, but we should be able to obtain enough material to prepare the libraries when the rearing starts again. Second, we plan to determine the influence of *Wolbachia* ( $\pm$  BEV-like) and/or B-vitamins on host gene expression in response to feeding, by comparing 4-week starved or fed females.

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<sup>18</sup> We cannot get developing and fertile  $W^-Vit^-$

### 1.2.3 - Metabolic exchanges and dependency between partners

As I presented before, bedbugs have established an obligate, nutritional, symbiosis with *Wolbachia*. A couple of studies have shown that *Wolbachia* mainly provides riboflavin and biotin to bedbugs (Nikoh *et al.* 2014; Moriyama *et al.* 2015), as other nutritional symbionts do in their strict hematophagous host (Duron & Gottlieb 2020). However, the genetic basis of this dependence and the global interactions between *Wolbachia* and *Cimex* have not been studied much to date.

BEV-like is a close relative to BEV and *Symbiopectobacterium purcellii*, a symbiont of the leafhopper *Empoasca decipiens*. These latter symbionts are larger in size than typical heritable symbionts, and contain numerous secretion systems and B-vitamins biosynthetic pathways (Degnan *et al.* 2011; Nadal-Jimenez *et al.* 2022). It is thus possible that BEV-like also retained these genes/operons that could play a role in the symbiosis with bedbugs. Because the genome of BEV-like is not described yet, we established a collaboration with Greg Hurst and Stefanos Siozios (University of Liverpool), who have run several sequencing projects (illumina and nanopore) to describe a torix *Rickettsia*, and who have by-product reads of BEV-like that we can use to try reconstructing its genome. If this draft genome is missing information, we plan to perform complementary sequencing on Malpighian tubules (where BEV-like resides (but not *Wolbachia*)) using a selective nanopore sequencing technology.

All together, we would like to characterize exchanges between *Cimex* and *Wolbachia*, *Cimex* and BEV-like, and *Wolbachia* and BEV-like. Indeed, microbial cross-feeding between *Wolbachia* and BEV-like could also occur and it is interesting to consider the microbial exometabolome (Douglas 2020), as described in sharpshooters, cicada or spittlebugs.

- [Metabolic networks](#)

The metabolic networks of the insect and symbionts are being reconstructed using a semi-automatic procedure developed at BF2i by Hubert Charles, Patrice Baa-Puyoulet, and Juan Carlos Pérez (INSA student). For now, predicted Enzyme Commission (EC) numbers and Gene Ontology (GO) terms collected by CycADS (Baa-Puyoulet *et al.* 2016) from *Cimex lectularius* genome (Rosenfeld *et al.* 2016), *Wolbachia wCle* genome (Nikoh *et al.* 2014) and preliminary sequences data from BEV<sup>19</sup> are processed with the Pathway Tools software (Karp *et al.* 2019) to infer enzymatic reactions and metabolic pathways. This semi-automatic pipeline consists of several steps:

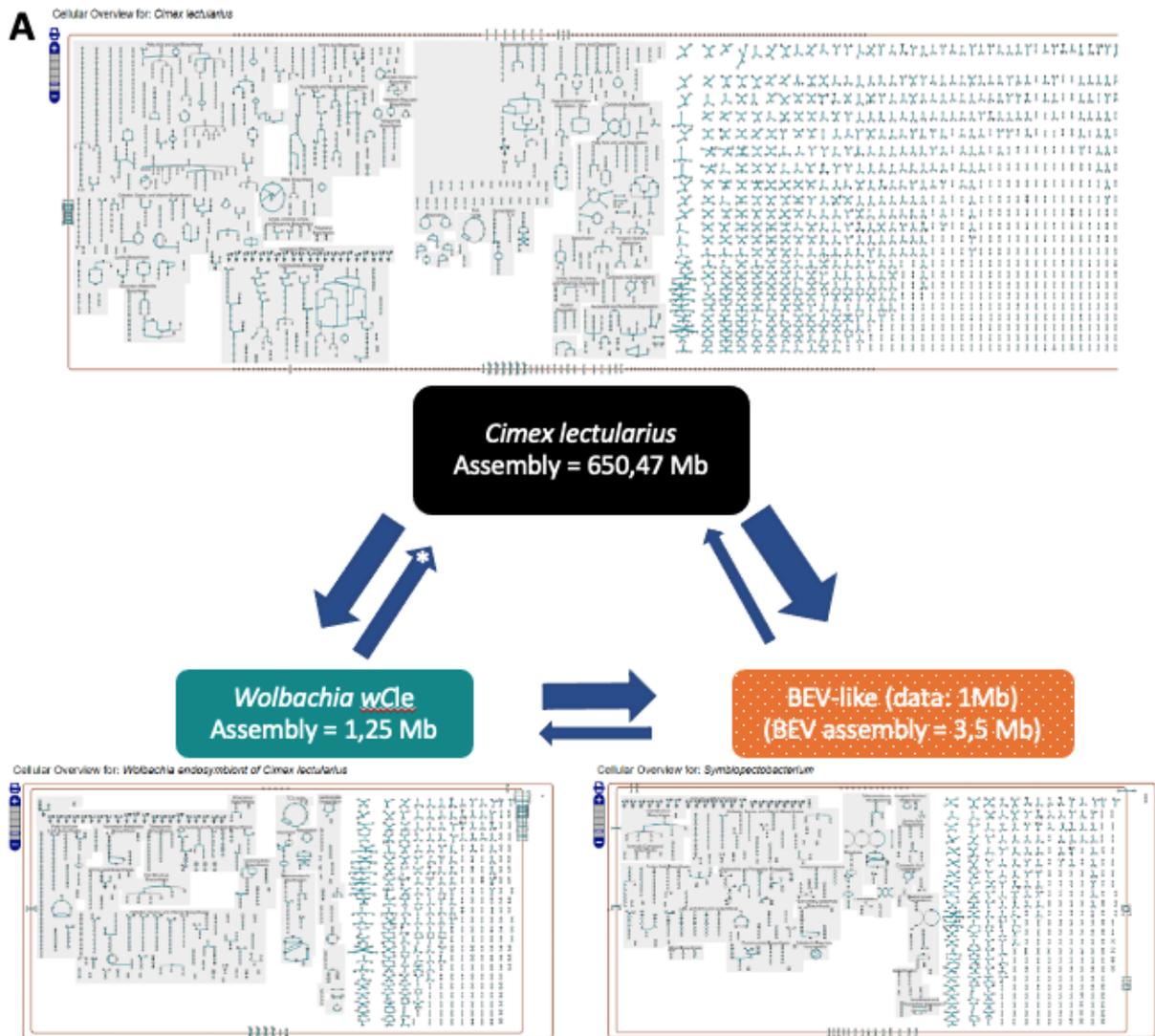
- 1) For each organism, reconstruction of metabolic pathways and analysis of essential metabolic pathways in a central metabolism category.
- 2) For each organism, identification of metabolites in metabolic pathways: precursors and dead-ends<sup>20</sup> of central metabolism.
- 3) Identification of external input (blood) compounds.
- 4) Identification of possible complementations between host/symbionts and symbiont/symbiont.

The data are implemented in the ArthropodaCyc (Baa-Puyoulet *et al.* 2016) and ArtsymbioCyc (<http://artsymbiocyc.cycadsys.org>) databases to reconstruct the CimleCyc (*C. lectularius*) and WolbachiaCyc metabolic databases, respectively. Outputs from these analyses are currently analyzed by Raphaël Jorge during his PhD project (**Figure 35**).

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<sup>19</sup> 1Mb of PoolSeq reads from a project developed by H el ene Henri, Chlo e Haberkorn and Julien Varaldi are currently used for a preliminary analysis. The pipeline will be run again as soon as the genome assembly based on data from the University of Liverpool is obtained.

<sup>20</sup> A precursor is a compound used but not produced, while a dead-end is a compound produced but not used.



**B**

	Compound	Common Name	Reactions	Pathway
1	Reduced-ferredoxins	a reduced ferredoxin [iron-sulfur] cluster	[ RXN0-884 ,  RXN0-884 ,  ISPH2-RXN ,  ISPH2-R...	[ NONMEVIPP-PWY ,  PWY-5121 ,  NONMEVIPP-PWY ,...
2	Butanoyl-ACPs	a butanoyl-[acp]	[ RXN-9515 ,  RXN-9515 ,  RXN-9516 ,  RXN-9516 ]	[ PWY-7388 ,  PWY-8279 ,  PWY-7388 ,  PWY-8279 ]
3	Octanoyl-ACPs	an octanoyl-[acp]	[ RXN-9526 ,  RXN-9526 ,  RXN0-947 ,  RXN-1303...	[ PWY-7388 ,  PWY-8279 ,  PWY0-501 , None,  PW...
4	RIBOFLAVIN	riboflavin	[ RIBOFLAVIN-SYN-RXN ,  RIBOFLAVINKIN-RXN ]	[ RIBOSYN2-PWY ,  RIBOSYN2-PWY ]

**Figure 35 | Construction of metabolic networks**

**A.** Maps represent overviews of metabolic pathways in *Cimex lectularius*, *Wolbachia wCle* and BEV-like (partial genomic data). Arrows between organisms (ex: #1 towards #2) represent candidate exchanges of metabolites (ex: dead-end of organism #1 = precursor of organism #2). The size of the arrow is proportional to the number of metabolites. **B.** Example of output of the pipeline showing 4 metabolites provided by *Wolbachia* to *Cimex* (arrow with white star).

Based on preliminary analyses, the metabolic pathways of *Cimex* seem to contain twice as many metabolites as those of the symbionts; overall 20% of metabolites are precursors and 20% are dead-ends. Exchanges of metabolites from *Cimex* to its symbionts are more frequent than the opposite, and symbionts can also exchange metabolites. As expected, *Wolbachia* does provide Riboflavin and Biotin to *Cimex*, but this network reconstruction suggests that BEV-like is also able to synthesize Riboflavin and to transfer it to *Cimex*. The reconstruction of these metabolic pathways and exchanges between partners (*Cimex*, *Wolbachia* and BEV-like) will be very helpful to target candidate pathways involved in the interaction between partners and in potential mechanisms of dependence.

- [Global transcriptomics and metabolomics](#)

In parallel with *in silico* analyses based on genomic data, we plan to analyze the molecular dialogue between partners *in vivo* and without *a priori*, both in terms of gene expression and metabolites.

Based on the literature, analyses on bedbug transcriptomes (Bai *et al.* 2011; Rosenfeld *et al.* 2016) indicate extensive transcriptomic rearrangements between the egg and the second nymphal stage, as well as during the nymph/adult transition. However, they only focus on the bedbug side (Rosenfeld *et al.* 2016) and no studies have so far looked at simultaneous changes in bacterial gene expression. To better decipher the molecular dialogue between the partners, we thus plan to conduct **Dual-RNAseq** (Westermann *et al.* 2017), a high throughput sequencing approach that allows the simultaneous analysis of the transcriptomic signatures of bedbug and both symbionts. We plan first to determine the **influence of blood feeding on the gene expression of both adult bedbugs and bacteria**. We will first focus on the adult stage to avoid any confounding effect of blood nutrition and molting, and will collect individuals a few days after feeding, to avoid the immediate response to blood ingestion. We plan to compare females, ovaries or bacteriomes between starved and fed bedbugs. Dual RNAseq will be performed on bedbugs using the NuGEN Ovation technology that allows the depletion of bedbug rRNA using specific probes and Illumina sequencing (2 x 100 pb). Data analysis will help to identify genes and cellular paths important for the symbiotic interaction during blood feeding and molting.

In order to characterize metabolic changes associated with blood feeding, we will complement the Dual-RNAseq analysis by a **comparative metabolomic analysis in fed or starved females**. A **whole metabolome screening**, which includes the quantification of main amino acids, carbohydrates, lipids, nucleotides, cofactors, and vitamins, will be performed by mass spectrometry linked with the largest reference library of known metabolites. Metabolomic data will be interpreted in light of the respective metabolic capabilities of *C. lectularius*, *Wolbachia* and BEV-like.

- [Targeted analyses and validation of candidates](#)

After datamining of the results of RNAseq, DualRNAseq, metabolic screen and metabolomic networks, we plan to test **the impact of interesting candidate genes/pathways/metabolites with regard to symbiosis** (*i.e.*, control or preservation of the symbiont inside of the bacteriome and/or metabolic exchanges) using **RNA interference (RNAi)** against specific genes. An engineer will be hired within the FBI program to set up the technics, based on already published protocols (Moriyama *et al.* 2016; Tsujimoto *et al.* 2017; Basnet & Kamble 2018). The Loss-Of-Function phenotypes will be screened at multiple scales: host survival after molting; adult size, fecundity, and survival after blood feeding; symbiont load and spreading. We will next develop a **more comprehensive functional analysis** of the most promising genes: nutrient supplementation experiments (*e.g.*, with vitamins B) or enzyme inhibition will allow to confirm the involvement of candidate genes in nutrient synthesis or exchange.

All together, these results will pave the way for developing prototypes of disruption methods. Such

methods could take advantage of the dependency of bedbugs to *Wolbachia* (and *BEV-like?*) for their nutrition and development, by inhibiting genes or pathways involved in the metabolite transfer between *Wolbachia* (*BEV-like?*) and bedbugs or in the molting process. Methods could also promote the elimination of symbionts (*via* antimicrobial peptide synthesis or inactivation of genes involved in maintaining symbionts), or at the opposite, the pathogenic hyperproliferation of symbionts (*via* the inactivation of host genes involved in its control).

More generally, these results may open another aspect of the biology of bedbugs, related to **co-symbiosis**. Indeed, the presence of multiple nutritional symbionts within a host is already observed in ticks or in leafhoppers (Wu *et al.* 2006; Buysse *et al.* 2021), and studying complementary or exclusion between symbionts, as well as potential mechanisms of replacement of symbionts is particularly fascinating.

### 1.3 - Other projects relative to bedbugs

#### 1.3.1 - Characterization of the 'oxidized' phenotype

As I described earlier, a major problem occurs in our rearing facility, and consists of the mass death of individuals a few days after blood feeding. This phenotype, which is probably due to a permeabilization of the gut membrane and the oxidation of the blood that reached all the tissues of the body, began in 2021 after the purchase of bedbugs from the CimexStore® company. It spread from population to population and eventually remained at more or less low levels, until the last year when we experienced very high mortality episodes. This pattern rather suggests the transmission of an infectious agent or the presence of toxic components in the blood during blood feeding. CimexStore® informed us that they experienced a strong outbreak (90%) before the shipment of the bedbugs, and that they suspect humidity to play a role in triggering the phenotype, especially in high density population. When we talked about this problem to other labs, they told us that they sometimes had such problems, but that it killed 5% minimum of the population (instead of sometimes 60% in our case). The addition of anticoagulants does not seem to be the main reason, since feeding is done directly on the human arms in O. Otti's laboratory, and this phenotype is still observed.

Digging into the literature, Chloé Haberkorn (CIFRE thesis supervised by Julien Varaldi and Fabrice Vavre) found that the salivary gland hypertrophy virus (GpSGHV, Hytrosaviridae) also causes high mortality in the tsetse fly *Glossina pallidipes* (Abd-Alla *et al.* 2021). Interestingly, this viral infection causes phenotypes ranging from no symptoms to death (Abd-Alla *et al.* 2021), with a variation in fitness costs, notably associated with a reduction in fertility. Indeed, GpSGHV infection has been shown to be associated with salivary gland hypertrophy and testicular degeneration (Kokwaro 2006). Another important feature is that GpSGHV increases in prevalence when tsetse flies are reared successively on the same blood meal in an *in vitro* system (Abd-Alla *et al.* 2021). As some latent, asymptomatic, viral infections are known to burst in certain abiotic conditions, such as temperature, hygrometry or density (Tanada & Omi 1974), and because of the transmission mode of GpSGHV, we decided to go dig the hypothesis of the infection of bedbugs by a pathogenic agent. Several experiments were run in parallel, which allowed us to adjust our rearing protocol to limit potential transmission. However, the fact that we do not harbor any BEV-free population limits our experiments and interpretations.

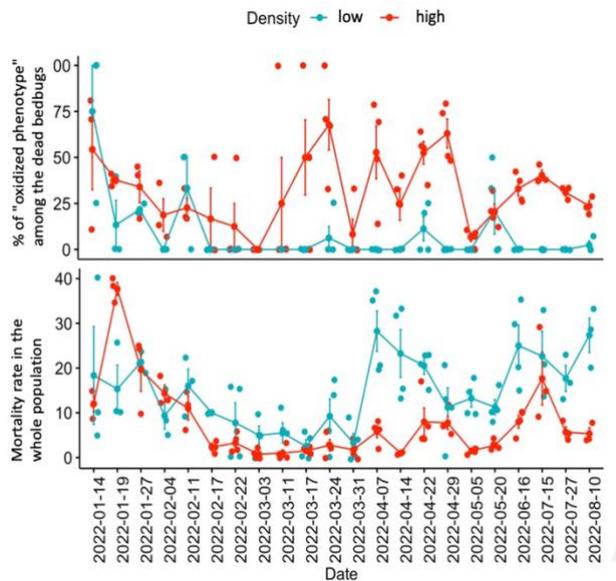
- [Influence of bedbug density on the emergence of the 'oxidized phenotype'](#)

We first wanted to test the impact of bedbug density on the frequency of mortality episodes. Elodie Rosinski, assistant engineer in charge of the rearing, thus split a S1 population into 2 regimes: a "low density" rearing with 15-50 individuals (mixed stages and sexes) per pot, and a "high density" rearing

with 100 to up to 900 individuals per pot (at the end of the experiment). Bedbugs were fed (1 feeder/replicate; 3-4 replicates/condition) and counted bedbugs every week for 5 months.

This experiment (**Figure 36**) shows that mortality events associated with the ‘oxidized phenotype’ were indeed more frequent in the high-density rearing condition, although we did not experience any major mortality event during the timeframe of the experiment.

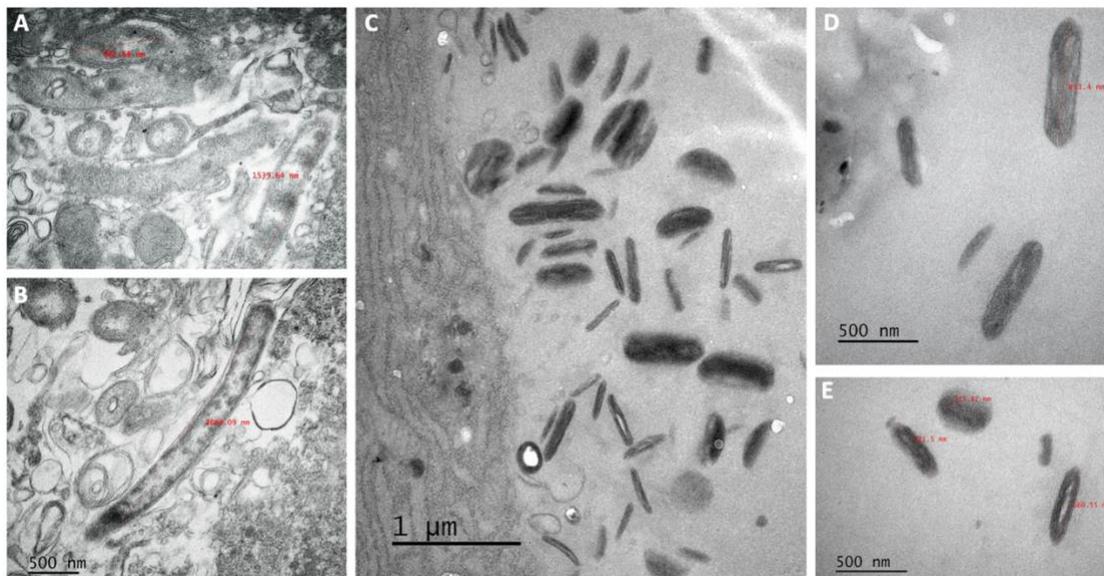
Contrary to our hypothesis, bedbugs from the low-density rearing condition survived less as compared as the ones from the high-density rearing, but they died without exhibiting any specific phenotype. We could explain this high mortality rate by non-optimal ecological conditions at low density. Indeed, bedbugs are gregarious and may maintain a certain level of humidity at high density, necessary for a good survival rate. New experiments testing for the role of aeration within the climate chamber (and thus local humidity) are underway.



**Figure 36 | Evolution of mortality rates and ‘oxidized phenotype’ prevalence under low (blue) or high (red) density rearing.** Mean  $\pm$  SE, and individual measurements per pot (n = 3-4 replicate pots/condition)

- Observation of salivary glands by TEM

We also investigated the presence of bacteria or viruses within bedbug salivary glands using Transmission Electronic Microscopy at the CTμ<sup>21</sup>. Indeed, many pathogens are transmitted through the saliva during blood feeding. I dissected salivary glands in samples that exhibited the ‘oxidized phenotype’ (just before they died) or did not exhibit it (but could still be infected asymptotically).



**Figure 37 | Salivary gland lumen observed by Transmission Electron Microscopy (TEM).** *Wolbachia*-like (A) and elongated rod-shape bacteria (B) in the tissues of the salivary gland. Rod-shape bacteria (C-E) in the lumen of the salivary gland.

<sup>21</sup> Centre Technologique des Microstructures, a multidisciplinary microscopy platform (with the help of L.Geai)



Among the RNA sequences (**Figure 38B**), she mainly identified contigs belonging to *Cimex lectularius* and *Wolbachia* wCle, and 3 viral contigs belonging to reovirus, partitivirus (and errantivirus).

*Alicagenes faecalis*, BEV-like and a reovirus seem to be interesting candidates, as close relatives have been involved in plant or insect pathogenesis (Oulghazi *et al.* 2021; Xu *et al.* 2022). She thus designed primers to amplify these candidates but did not manage to specifically amplify *Alicagenes faecalis* nor the reovirus in fresh samples. She could only amplify BEV-like in bedbugs expressing oxidized and non-oxidized phenotypes.

As mentioned above, we also suspect a link between the presence of BEV-like and the 'oxidized phenotype', apart from a nutritional or excretory role. Raphaël Jorge will now explore in depth the possible impact of BEV-like on bedbug traits, determine its prevalence in natural populations, and a potential correlation between its density and the onset of the 'oxidized phenotype'. We are thus planning to quantify BEV-like by qPCR and observe gut epithelium by TEM in bedbugs alive or starting to express the oxidized phenotype. Because we observe the 'oxidized phenotype' in aposymbiotic populations, the impact of the antibiotic treatment in BEV density will tell us whether BEV-like is a good candidate for the pathogeny observed in our rearing.

Overall, these lines of research are helping us to better understand the basis of this oxidized phenotype. In the short term, this will enable us to improve rearing quality and productivity, and in the longer term to envisage a potential new biocontrol agent against bedbugs.

### 1.3.2 - Interaction *Wolbachia* / insecticides

Gut or intracellular (*e.g.*, *Wolbachia*, *Rickettsia*, *Burkholderia*, *Arsenophonus*) symbionts have been shown to degrade various types of insecticides, and to induce insecticide resistance in a variety of insects (*e.g.*, planthoppers, fruit flies, beetles, cockroaches, mosquitoes, moths), including in insects belonging to the same order than bedbugs (aphids, whiteflies) (Pietri & Liang 2018; Blanton & Peterson 2020). The presence of endosymbionts can instead increase susceptibility to certain insecticides in some insects, notably by impacting negatively host fitness (Skaljac *et al.* 2018), through various costs related to metabolism or immunity (Pang *et al.* 2018). Therefore, the impact of symbionts on the response to insecticides depends on the balance between the benefits linked to the biodegradation of the insecticide and the costs linked to the hosting of symbionts and the expression of the detoxification mechanisms.

On the other side, insecticides themselves or insecticide resistance genes can have an impact on symbionts, notably on symbiotic density. For instance, *Wolbachia* density was initially higher in insecticide-resistant strains of mosquito than in susceptible strains (Berticat *et al.* 2002), suggesting looser control of the bacterial population and increased cost in resistant strains (Duron *et al.* 2006). Interestingly after 50 generations, *Wolbachia* density in the resistant strains was similar to that of susceptible strain, suggesting that mechanisms of control can evolve rapidly (Echaubard *et al.* 2010).

Within the FBI ANR project, we thus plan to explore the **reciprocal interference between insecticides and *Wolbachia***, that is, to determine whether *Wolbachia* modulates the resistance to insecticides, and conversely whether the presence of insecticides impacts *Wolbachia* density and potentially the nutritional symbiosis.

- To determine if the disruption of the symbiosis makes bedbugs more susceptible to insecticides, or if *Wolbachia* expresses insecticide detoxification mechanisms that increase resistance, we plan to compare symbiotic and 'aposymbiotic + B-vitamins' individuals at different developmental

stages after insecticide treatment, and to measure the molting, survival rates, and cuticle thickness. These tests will be performed in insecticide susceptible and resistant lines. Transcriptomic analysis by RNAseq could also inform on the molecular resistance mechanisms mediated by *Wolbachia*, such as insecticide detoxification or any resistance mechanism mediated by the control of host gene expression by *Wolbachia*.

- To determine if insecticides impact *Wolbachia*, notably its density, and could then destabilize the nutritional symbiosis and the bedbug physiology, we will first quantify the direct impact of insecticide treatments on bacterial density (using qPCR) on susceptible and resistant strains, using different concentrations of insecticides (starting from sublethal concentrations). We expect an increase in bacterial density if the insecticide disrupts host mechanisms that control bacterial density. In this case, the expression of genes involved in bacterial control will be quantified by qRT-PCR. Alternatively, a decrease in bacterial density is expected if the insecticide directly impairs *Wolbachia* physiology or the bedbug cellular environment in which *Wolbachia* resides. In this case, host traits directly influenced by *Wolbachia* should also be impaired. As Riboflavin is an essential cofactor in the glutathione redox cycle and involved in the synthesis of P450 detoxifying enzymes (Kennedy 2016), we expect differences in survival/molting. Focusing more generally on the impact of insecticides on host metabolism, we plan to measure the expression of candidate genes involved in metabolic exchange in presence/absence of insecticide.

## Chapter 2 - Science & society

Fundamental research provides the foundations for understanding the species that surround us and the ecosystems in which they live. But we also live in a society that has an impact on our environment. I therefore try, as part of my research, to think about the consequences of my actions and my research, to be as aligned as possible with the values I defend, and to try to limit my negative impact on the environment as much as possible, despite the complexity of the challenge. I have therefore committed myself to various aspects (societal, ethical, and environmental issues) that I will present below.

### 2.1 - Bedbugs: a public health problem

#### 2.1.1 - Collective expertise report on bedbugs at ANSES

The Ministry of Ecological Transition and the Ministry of Solidarity and Health have issued a referral in connection with the publication of a *Plan d'action interministériel de lutte contre les punaises de lit*<sup>22</sup> in March 2022. Through this action plan, the Government plans to develop a coherent interministerial strategy to fight bedbug infestations, based on better public information, support for households in dealing with infestations, development of a national observatory of the phenomenon, and the consolidation of scientific and technical expertise. In this context, the *Direction Générale de la Santé* (DGS) and the *Direction Générale de la Prévention des Risques* (DGPR) have commissioned the *Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail*<sup>23</sup> (ANSES) to provide scientific expertise on:

- the characteristics of bedbugs found in France, particularly in terms of resistance to insecticides, and their potential for transmitting infectious agents;
- the understanding of the dynamics of the presence and proliferation of bedbugs (bio-ecological, physical, technical, socio-economic, demographic, cultural, geographical factors, etc.);
- the control methods against bedbugs, to determine the most effective methods in terms of preserving the health of those exposed (during and after use) and their effects on the environment;
- the identification of the benefits and potential risks associated with the various control methods available, with:
  - an analysis of the risks to human health and to the environment, particularly for chemical control methods and their associated impacts;
  - an analysis of the benefits in terms of public health (psychological damage, quality of life).
- some recommendations to prevent infestations.

A 'Bedbugs' working group was set up for 15 months, bringing together complementary expertise: biologists (entomology, ecology, microbiology, evolutionary biology), physicians (medical entomology, dermatology), and economists. Within this group, I thus participated in the writing of the ANSES collective expertise report, published in July 2023, and was particularly involved in the writing of the chapters 3 (Biology and behavior of bedbugs), 7 (control methods), 10 and 11 (conclusions and recommendations).

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<sup>22</sup> interministerial action plan to combat bedbugs:

[https://www.ecologie.gouv.fr/sites/default/files/22012-punaise\\_plan%20de%20lutte\\_DEF.pdf](https://www.ecologie.gouv.fr/sites/default/files/22012-punaise_plan%20de%20lutte_DEF.pdf)

<sup>23</sup> ANSES: French Agency for Food, Environmental and Occupational Health & Safety. It is a public administrative body reporting to the Ministries of Health, the Environment, Agriculture, Labor, and Consumer Affairs.

Although some members of the working group have very strong opinions and the working methodology could be optimized, the exchanges within the group have provided me with a better understanding of the reality on the ground and the political and economic issues at stake. Interviews with various players, such as social housing organizations, hotel trade unions, citizens' associations and the CS3D trade association, were particularly instructive. Building and analyzing general population surveys has also been very interesting, and has made it possible to fill, in part, the gap in information on the incidence and psychological and economic consequences of bedbugs in France.

Overall, this report strongly emphasizes the importance of recognizing bedbug infestation as a public health problem and to set up an observatory. It is essential to treat infestations as quickly as possible, starting with mechanical control (heat, freezing, vacuuming), rather than systematically resorting to the use of insecticides (especially by non-professionals). Upstream, it is crucial to inform and train the general public and non-insecticide-using professionals (housekeepers, caretakers, nurses, etc.), and to develop public policies in this area, particularly for low-income populations.

### **Related publication:**

Rapport d'expertise collective (président : Lazzari C ; Membres : Braquart-Varnier C, Dalmas L, Delaunay P, Izri A, **Kremer N**, Lacaze I, Pecquet C, Verheggen F ; Coordination scientifique : Fite J, Fiore K, Hily E, Jaffal A, Larousse A). Les punaises de lit : impacts, prévention et lutte (2023). Saisine 2021-SA-0147. Maisons-Alfort : Anses, 258 p.

Rapport d'expertise collective (avec avis de l'ANSES sur le rapport) :

<https://www.anses.fr/fr/system/files/BIOCIDES2021SA0147Ra.pdf>

Annexe 4 (enquête menée auprès de l'Union Sociale pour l'Habitat) :

<https://www.anses.fr/fr/system/files/BIOCIDES2021SA0147Ra-Anx.pdf>

#### 2.1.2 - Working on bedbugs

One important aspect of the problem of bedbug infestation is social. Indeed, while the probability of being infested is similar in low- and high-income populations <sup>24</sup>, the outcome of an initial infestation is very different depending on the social class: high-income population will eradicate the infestation very quickly, while low-income population will not have the resources to limit the initial infestation, which will spread and become more complicated to treat.

Social inequalities are rarely taken into account in the fight against bedbugs, even though they play a major role. In this respect, it would be interesting to quantify the risk of infestation/dispersal, efficiency of detection, the resources available, and the access to psychological and medical care within different social classes. It would also be interesting to quantify the influence of social classes on the perception, use and effectiveness of different control methods (especially mechanical and chemical), and the impact of these uses on the evolution of insecticide resistance and on the migration of bedbug populations.

In order to go further in this direction, we have started, together with Fabrice Vavre, to contact people within the *Contrat local de Santé* <sup>25</sup>, e.g., Lyon Urban Hygiene Department, and the association *Le*

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<sup>24</sup> However, the reasons are very different: in low-income populations, the main sources of infestation are high occupancy rates and recourse to the second-hand market, while in high-income populations, infestations are mainly due to travel and the rental of lodgings/hotels.

<sup>25</sup> Local Health Contract (<https://www.lyon.fr/le-contrat-local-de-sante>), which is a tool for coordinating local players on global health issues.

centsept<sup>26</sup>, to discuss and start creating a network approaching these questions and to link social, medical and ecological/genetical approaches. Eventually, the new initiative SHAPE-Med@Lyon, which aims to develop transdisciplinary health research based on a "One Health" approach, could promote and fund research projects on bedbugs, at the frontiers between humanities, medicine, and biology.

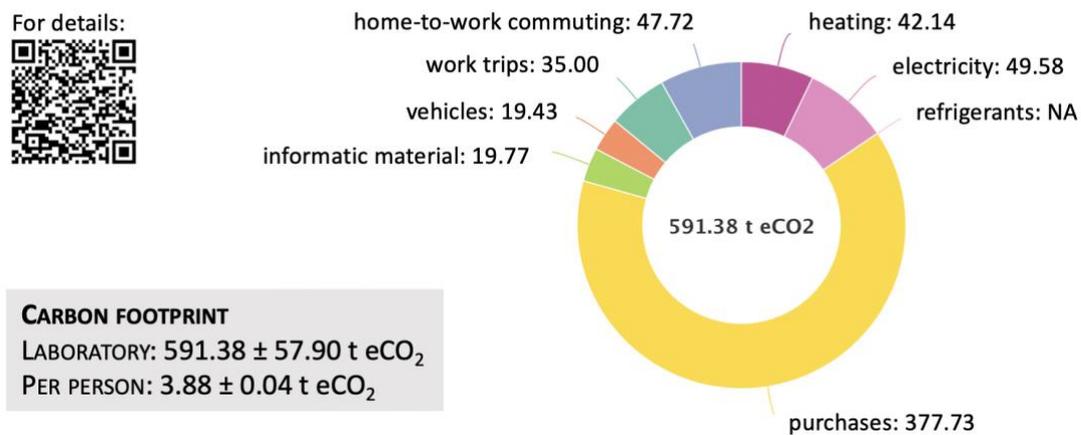
## 2.2 - Environment and ethics

### 2.2.1 - Environmental footprint

Climate change is on the march, and one of the most important ways of limiting it is to reduce our carbon footprint. This approach limits our individual and collective freedom, and it is therefore important to estimate our footprint as accurately as possible in order to target the most effective levers.

Together with the LBBE working group 'environmental footprint' and students from INSA Lyon, we have estimated the laboratory's carbon footprint in 2021, and plan to update it every year. For this purpose, we used the tools proposed par the *Labos 1point5*<sup>27</sup>, and set up anonymous pools to determine the information (notably on home-to-work commuting) that we did not have access through our institutions. GES tools are open-source tools for calculating the carbon footprint and constructing the laboratory regulatory greenhouse gas balance sheet (BGES). These tools help to identify levers for action to reduce the impact of research activities on greenhouse gas emissions, and enable scientific studies to be carried out on the carbon footprint of French public research.

Concerning the LBBE, we found a carbon footprint of  $591 \pm 58$  t eCO<sub>2</sub> in 2021, which corresponds to  $\sim 3.8$  t eCO<sub>2</sub> per person (Figure 39). This number is underestimated, as refrigerants were not integrated in the calculation, and corresponds to around 15350 km by car. The main consuming item is purchases, which include laboratory equipment and materials for experiments.



**Figure 39 | Carbon footprint of the Laboratory of Biometry and Evolutionary Biology (LBBE) in 2021.**

The footprint was estimated using GES tools from the Labos 1point5. NA: data not included in the estimation.

<sup>26</sup> <https://www.lecentsept.fr/>, which support projects with a social or environmental impact in consolidating their development and cooperation strategy (public, private and social economy players) in the Lyon area. One of their 'social innovation lab' is dedicated to bedbugs.

<sup>27</sup> Research group supported by CNRS, INRAE, ADEME and INRIA, whose aim is to better understand and reduce the carbon footprint of research activities: <https://labos1point5.org/>.

Currently, the carbon impact of a French citizen is around 8.9 t eCO<sub>2</sub> in 2021, and France's commitment at COP21 is to limit emissions to 2 t eCO<sub>2</sub> by 2050 (including private and professional activities) <sup>28</sup>. Compared to other laboratories of biology, the carbon footprint of the LBBE is lower, but we still need to define and apply levers for action to reduce it. A small number of laboratories have begun to set up initiatives, which gives us an idea of the feasibility and acceptance of these actions. The next step in the LBBE is to define a common reduction target and a deadline, as well as a procedure for validating (or not) actions (*i.e.*, quorum, percentage of acceptors) that will be proposed. Then, it will be necessary to take stock of the present situation to determine which levers would be the most effective at LBBE (*e.g.*: equipment purchasing policies, distribution of IT purchases, duration of warranties, use of transport during work trips...), and to brainstorm about possible actions that we could vote and apply. An important step is to inform people on their implementation and impact, by establishing scenarios and estimating the associated reductions. As it is complicated to change the core of our activities immediately, we will start with measures that are easy to implement. A more general reflection on our choice of research topics and the way we conduct them is underway, with the support of specialists in the field whom we have invited to give seminars. In the meantime, we have started with small "day-to-day" actions that reduce our ecological impact (*e.g.*, selective recycling and compost, information on the carbon impact of food, use of recyclable cups in the laboratory, zero-waste picnics, ...).

#### 2.2.2 - Ethics in Science and FAIR principles

Ethical practices are necessary in scientific research to guarantee the quality and integrity of the science on which research is based. They contribute to the development of knowledge, technical progress, and values in our society. Many initiatives promote ethics in science and try to limit potential bias that arose in the scientific community. In 2016 was published a Guidelines to improve the management of scientific data (Wilkinson *et al.* 2016). This guideline was structure around 4 main values: **F**indability, **A**ccessibility, **I**nteroperability, and **R**euse of digital assets, described by the GOFAIR website <sup>29</sup>. To summarize this approach, metadata and data should be easy to find and be accessed, integrated with other data in an interoperable way for analysis, storage, or processing. In order to limit the waste of information and to optimize the efforts required to obtain data, (meta)data should be well-described to be reused for other questions or replicated. In this context, I try (in my own way) to contribute to this philosophy:

- In order to facilitate the reporting of results during experiments, I set up an **electronic laboratory notebook** in the lab, with the help of Stephane Delmotte. As this approach was not yet studied at CNRS or UCBL level in 2020, I explored the various electronic laboratory notebook applications and chose ElabFTW, developed by Nicolas Carpi at the Institut Curie (Deltabolt) <sup>30</sup>. Indeed, it is a secure, free opensource and compliant system (A+ rating on Mozilla's Security Observatory) that can be implemented on a secure server within the laboratory using Docker containers (tool durability). It allows an efficient tracking of experiments (markdown, integration of images, files, code extracts) and contains a powerful, flexible database for lab management (protocols, storage). In addition, this tool is designed for multiple user management and possess a reliable time-stamping system of experiments. I thus defined a user charter for the use of the LBBE-CLE electronic notebook and offer

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<sup>28</sup> <https://www.ecologie.gouv.fr/strategie-nationale-bas-carbone-snbc>.

<sup>29</sup> <https://www.go-fair.org/fair-principles/>.

<sup>30</sup> <https://www.elabftw.net/>.

regular training courses together with Nicolas Carpi. Since 2023, the CNRS has also chosen this tool and offers it to its units.

- I promote **open data** for my research (HAL, BioRxiv, Zenodo preprint depository, SRA), and my publications. In particular, I support the initiative developed by **Peer Community In (PCI)** <sup>31</sup>, which aims at processing scientific preprint based on peer reviews, and to deliver free recommendations when publications reach a certain quality level. This label ensures the quality of a free preprint, and limits the runaway success of publishing houses specializing in the scientific press, especially predatory journals. In this respect, I am a recommender of PCI Evolutionary Biology and PCI Zoology, and published two papers (Bénard *et al.* 2021; Monnin *et al.* 2021) in PCI Evolutionary Biology (now gathered in PCI journal). I also published a paper with **Camille Noûs** <sup>32</sup> as a co-author, to symbolize my attachment to collaborative research and integrity in a context of French research funding policy strongly oriented towards individuals, and drifting towards publication race practices.

### 2.2.3 - Well being at work & Equality/diversity at LBBE

In all the laboratories where I did my research, I appreciated the scientific quality of the host teams, the freedom I was able to have in my work, and the helpfulness of my colleagues. Indeed, I believe that a pleasant, collaborative working environment is the key to professional fulfillment. That is why I am involved in the "**Quality of Life at Work**" working group. This group was asked to investigate well-being. In this context, we have set up a survey on the well-being of thesis students, a series of internal and external seminars on supervisory aspects, and a discussion on support/mediation measures. It is also involved in more practical actions, such as furnishing the laboratory rooms, and in particular the social rooms (following the renovation work that has just been carried out), or setting up photo/art competitions to decorate the corridors.

I also promote the role of women in research, and diversity in general. I've been lucky enough to be mentored by people who have supported me and given me plenty of room to build my own "niche". I would like to pass on the message that everyone has a place in science, provided it is of high quality, and that we must defend our positions wherever we wish to be. As part of the "**Equality / Diversity**" working group that has just been set up, we began by assessing the participation of speakers during meetings (according to the gender and status of participants), and to encourage women and non-permanent staff to ask questions.

## 2.3 - Outreach

Although I do not feel extremely comfortable speaking in public, I make a point of disseminating the knowledge I have been lucky enough to acquire. To this end, I have spoken at schools (*Declics* <sup>33</sup>, *Mercredis de Lyon 1 Sciences* <sup>34</sup>), but also for the general public. In particular, I have presented my biological model systems during science festivals (*Darwin days* in Madison, French-American Science

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<sup>31</sup> <https://peercommunityin.org/>

<sup>32</sup> <https://www.cogitamus.fr/camilleen.html>

<sup>33</sup> Dialogues between Researchers and High School Students to Interest Them in the Construction of Knowledge: <https://www.cerclefer.org/fr/declics/>

<sup>34</sup> Lyon 1 Sciences' Wednesdays: welcome high school students (1ère) via the Student Orientation and Professional Integration Service (SOIE)

Festival in Chicago, *Fête de la Science* in Villeurbanne), participated in the development and animation of a Street Science application <sup>35</sup> in the framework of the International Symbiosis Society meeting, or presented my research in the *Université ouverte (UCBL)* or in a societal context with *Pint of Science* <sup>36</sup>. I like to see the curiosity of kids and adults and discuss with them about various subjects and values in science. Because I like connections between art and science, I took the opportunity to present my research interests and projects to a producer of the *n<sup>ième</sup> compagnie*, who created the play '*Insectes: histoire en (dés)équilibre*' <sup>37</sup>, and to meet with the audience at the end of the play. It was very interesting to see the concepts that had been retained from the scientific exchange, but above all how to make a message funny, while making the audience think about the place of insects in our society, the environmental impact of the control methods used and the fragility of this ecosystem.

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<sup>35</sup> For the print & play version: <https://streetscience.fr/1251-2/>

<sup>36</sup> <https://pintofscience.fr/about/>

<sup>37</sup> <https://niemecompanie.fr/insectes-histoire-en-desequilibre/>;  
teaser: <https://www.youtube.com/watch?v=Vlyd2ktSLcw>

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