POLYTECH TOURS

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Individual internship report – 5th year

Role of the microbiota in the adaptation of the insect pest *Drosophila suzukii* to different host fruits

INRAE, CBGP

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2022-2023

INRAE la science pour la vie, l'humain, la terre

CBGP Centre de Biologie pour la Gestion des Populations

INTERNSHIP CONTEXT

Structure

The centre of biology for populations' management (CBGP) is a research laboratory in biology. It is a mixed unit of research (UMR) which is an administrative type of research laboratory that is partially supported by the national research institute for agriculture, nutrition, and environment (INRAE) and partially by other organizations, in terms of funds, academic and non-academic staff, and other type of support, based on a renewable multi-year contract.
The other organizations related to the CBGP are the institute of research for the development (IRD), the international cooperation centre in agronomic research for the development (CIRAD), and the engineering school in agronomy of Montpellier (Montpellier SupAgro).
The mission of the CBGP laboratory is to study harmful species – insects, acarids, small mammals – in their diversity, structure, and ecology, in order to predict their evolution in a global context. Around 80 peoples are working in the laboratory permanently – researchers, engineers, technicians, assistants, etc. – in a "thematic axis" depending on their research subject. There are three axes currently in the CBGP: (1) characterization and evolution of biodiversity, (2) evolutive ecology and genetic of pests, (3) ecology and evolution of zoonoses. The supervisor of my master internship, Nicolas O. Rode (INRAE researcher), is working in the second axis on the biological model *Drosophila suzukii*, an Asiatic small fly invading berries cultures.

Mission

The objective of my internship was to test a hypothesis on the ability of *Drosophila suzukii* to infest different fruits successively. The precise subject is to test for the role of the microbiota in the adaptative phenotypic plasticity of *D. suzukii* to its different host fruits. In other words, to test the presence of a fruit-specific microbiota which lead to a higher and more efficient reproduction on the corresponding fruit rather than on another fruit.
The subject of my internship is part of the research program "Adaptation of the Polyphagous Pest *Drosophila suzukii*: Role of the interactions between Fly Genotype, Microbiota and Host Fruit" (ADAGIO). It is a 4-year project financed by the Research National Agency (ANR). The overarching goal of ADAGIO is to better understand how fruit-specific microbiota mediate the genetic and plastic responses of polyphagous insects to their host plants.

My internship was designed in three parts. First, two to three months to do the bibliography on the subject, learn the experimental technics, prepare the experiment (size, tests, needs, etc.) and do the experimental pre-steps. Then, realizing the experiment for one month and a half. And finally analyzing the results and writing my report for another one month and a half. The main idea of the experiment was already decided when I started my internship, it was then more precisely designed. For the needs of my internship, I have learned from the technicians of the project, mainly C. Deschamps, the *D. suzukii* population's management technics in the dedicated CGBP's laboratory (maintenance of the flies' population, preparation of the media needed, etc.).

My internship didn't have proper "deliverables" other than the results of my experiment that I present in a scientific article structure further down, as is it part of a bigger research subject. Besides, I have produced some protocols for the different experimental pre-steps and some oral presentations for meetings (an ADAGIO appraisal and presentation of the objectives of my internship to the axis staff).

Feedback on the experience

Working in a research laboratory in ecology and biology was a new experience for me. I discovered how a research project is structured at the laboratory scale, i.e., how the subject is comprehended through its different steps, but also at the human scale, i.e., how the team working on the subject is articulated. Doing my internship in the working team of Nicolas O. Rode composed of laboratory technicians, engineers, and international researchers, on the recently launched research project was the perfect situation to understand and comprehend all of these structural aspects.

Early in my internship I've been included in the general dynamic of the laboratory and in the dynamic of the working team, by participating to meetings, learning the technical methods, exchanging with people, etc. It permitted me to be in a coherent work dynamic with my internship subject, within the bigger working context of the laboratory.
The internship approach and methods executed were well structured and updated throughout the whole period thanks to the initial planning and to frequent planning adjustment meetings with my supervisor, and if needed with the other members of the research project who helped me.
The "DISCUSSION" part of my report is a critical review of the produced results, it also includes some change trails for a further study of the subject.
Finally, this internship was a revealing experience for my working ambition. The research field in evolutionary ecology is a working context in which I completely picture myself in. I think this internship experience is in the continuity of my engineer formation because I have concretely learned to work in a pluri-disciplinary international team on a project. This experience was so interesting and rewarding that I will continue with a thesis, supervised by Nicolas O. Rode and Benoit Facon. The subject is in the continuity of my internship subject, on the role of the gut microbiota of *D. suzukii* in its adaptation to its different host fruits.

GLOSSARY

Definitions of the words in the document followed by a ' * '.

Axenized
Organism made free from micro-organisms germs through the process of axenization (for instance, bleaching of insects' eggs) (Larousse dictionary online [1]).

Local Adaptation
Natural evolutionary mechanism whereby individuals of the same species living in a given area (a population) evolves to stay adapted to their local environment (Williams 2008). Compared to other individuals of the same species living in a different area, they are better suited to the considered environment (able to live better and longer).

Fitness
Measure of the contribution of an individual in its population, it corresponds to its reproductive success. Commonly it is the number of female offspring produced by an adult female in a given population (Maynard Smith 2004).

[1] www.larousse.fr

16S rRNA gene for sequencing
Sequence of genes coding for genetic information of procaryotes. It is used for phylogenetic studies because it evolved not much through time which permits to rebuilt evolutive history of organisms by comparison, and because it is highly conserved between different species of bacteria and archaea (Coenye and Vandamme 2003).

Gut Microbiota
Community of micro-organisms located in the digestive tract of an organism. It can be composed by bacteria, archaea, viruses, protozoa, and anaerobic fungi and this composition may vary over time (Macke et al. 2017).

Phenotype
Set of traits of an organism (morphology, physiology, behaviour, etc.) resulting of the expression of its genetic information and the influence of environmental factors (Oxford Dictionary online[2]).

Pupae
Intermediate development stage for Diptera insects between the larval stage and the adult stage (Triplehorn, Johnson, and Borror 2005). It is represented in the life cycle scheme in Appendix 6.

Reciprocal Common Environment Experiment
Experimental design aiming at testing the performance of different populations across different environments that resemble their environments of origin to test whether each population is phenotypically adapted to its local environment (Olazcuaga et al. 2021).
It consists in introducing individuals from a population originating from an environment A, into either artificial environment corresponding to different environments including the origin one (A, B, C) to assay their performance. For instance, here with A = forest, B = field, and C = plains.

Selection (natural selection)
Mechanism of evolution which depict the survival and reproduction rate of individuals depending on their phenotype (Darwinian definition). Individuals with certain beneficial variants of traits tend to reproduce and survive more than other individuals, inducing an evolution in the population.

> *Diversifying selection*
> Type of natural selection which tends to increase the variance the population mean of a given trait. It favours extreme phenotypes over intermediate (stabilizing selection), which leads to a potential division into two distinct groups.
>
> *Stabilizing selection*
> Type of natural selection which tends to stabilize the population mean of a given trait on a non-extreme value. It favours the intermediate phenotypes instead of extreme variants (diversifying selection).

[2] www.oxfordlearnersdictionaries.com

TABLE OF CONTENT

INTRODUCTION

The ecological niche of a species is the set of biotic and abiotic factors which define an area where the species can reproduce and survive. As environmental conditions often change over time and space, organisms may evolve by natural selection to stay “adapted” to the environment.

Adaptation is defined as the evolutionary process whereby an organism becomes better able to live in its habitat(s) thanks to a more suited phenotype*. Phenotypic adaptation is driven by two main mechanisms: genetic shifts through the increase in frequency of selected variants resulting from natural selection (Darwinian evolution), and plastic shifts resulting from environmental influence on individual development (adaptative phenotypic plasticity; (Lande 2009). In other words, genetic adaptation results in the selection of certain alleles, while adaptive phenotypic plasticity is made by shifts in development and behaviour, without genetic changes (Price, Qvarnström, and Irwin 2003).
The main advantage of plastic shifts over genetic shifts, is to provide organisms a faster response to an environmental change and it may also be reversible across generations. Thereby, multiple factors may be a source of phenotypic plasticity, such as learning, temperature variations or nutrition. For instance, wood ducks present a plastic adaptation of the size of their intestine which varies throughout the year and is positively correlated with the fibre content in their diet (longer intestine in the fall corresponding to higher dietary fibre; (Drobney 1984). However, understanding the developmental mechanisms responsible for adaptative phenotypic plasticity is not a closed subject. Although theoretical models predict a bigger plasticity for species living in changing environments, whether genetic variation in plasticity is driven by stabilizing or diversifying selection* and whether the strength of such forces remains constant through time, remain open questions (Becker et al. 2022).

Generalist phytophagous insects are great examples to investigate adaptative plasticity because they are specially adapted to their feeding plants. As generalists, they are able to feed in a large range of resources (contrary to specialists which have restricted diet). As phytophagous their resources are only constituted of living plant matter or the products of a plant (Bernays 2009). Thus, those species are able to comprehend multiple environments through their life cycle, which rest upon natural mechanisms.

Here, we assess the potential role of the gut microbiota* of phytophagous generalist insects as a plastic evolutionary force. The commonly designed-as-main function of the gut microbiota is digestion, but we know for more than two decades that it is also interacting with the host immune system, the energy harvest and storage, diseases, or even behaviour (Clemente et al. 2012). The gut microbiota may contribute significantly to the host’s adaptative phenotypic plasticity.

Our biological model is the Diptera *Drosophila suzukii*, also known as the spotted-wing drosophila. It is an invasive species originating from Asia able to feed on a wide range of host plants (more than 17 plant families; (Olazcuaga et al. 2022)). *D. suzukii* is a well-suited model to understand the role of phenotypic plasticity as a generalist phytophagous insect, but also as an invasive species. In fact, it has been highlighted that invasive species demonstrate significantly higher phenotypic plasticity than non-invasive species but not necessarily liked to a fitness benefit (Davidson, Jennions, and Nicotra 2011). The invasive success of the species may potentially be increased by phenotypic plasticity. Besides, the species can persists throughout the year in a given location on a succession of distinct host fruits, each one being available for one to four generations of *D. suzukii* (Kenis et al. 2016). In addition, its short life cycle (20-30 days) is an advantage to rapidly increase the fitness* of the species between the first and the last generation colonizing a particular fruit.

In a recent study L. Olazcuaga et al. (2022) performed a reciprocal common environment experiment* and found a pattern of local adaptation. Offspring performance was indeed higher in the medium corresponding to the fruit of origin than in media corresponding to alternative fruits. Our study frame enters in the continuity of this work by testing if the gut microbiota is responsible for the pattern of local adaptation observed by L. Olazcuaga et al. (2022) through adaptive phenotypic plasticity.
Indeed, studies suggest a local adaptation of this pest to its host fruit driven by phenotypic plasticity through the gut microbiota, in particular because larvae deprived of their microbiota have a very restricted growth. It even has been highlighted that, for studied diet, some key proteins required for the development of the fly are provided by the gut microbiota (Bing et al. 2018; Macke et al. 2017). So, we want to test the hypothesis assessing that gut microbiota appear as a fitness enhancing parameter through a beneficial fruit specialization.

The objective of this study is to test the role of the gut microbiota in *D. suzukii* adaptative ability to its different host fruits (blackberries, cherries, or strawberries).
My working hypothesis is that larvae acquire their gut microbiota in the fruit they infest, which later allow them to grow better in that fruit than in a different fruit (higher success of larva to become a pupa, and pupa to become a fly). In other words, my objective is to test for the beneficial effect of the fruit-specific gut microbiota in the development of *D. suzukii* larvae in a given fruit (proportion of larvae turning into pupae*, and then into flies).
To test for this pattern, we performed a reciprocal common environment experiment and tested whether pupariation rate and emergence rate were higher for the larvae and pupae developed in the same type of fruit they originated from. We realized the experiment two times (two batches).

MATERIAL & METHOD

Laboratory maintenance of the flies

To investigate the role of the gut microbiota in the larval performance of *D. suzukii* (pupariation rate and fly emergence rate) in different host fruits, we used two laboratory populations which have been maintained for more than one hundred generations in the laboratory (Olazcuaga et al 2021). The ancestral population was sampled in the wild on different fruits and later separated into different laboratory populations maintained on artificial media made either of cherry or strawberry purée (CEC and FRA populations respectively). The two laboratory populations has been maintained on a three-week life cycle (Olazcuaga et al. 2021). Flies were kept in tubes, under constant conditions of humidity (60±2%), temperature (21±2°C) and day/night light cycle (16 hours of light and 8 hours of dark). One tube contains 20 adult flies, with a sex ratio around one.

As the laboratory populations have been maintained in the same conditions for many generations (Olazcuaga et al. 2021), we hypothesize that their microbial community composition remains stable across generations, with a potential loss of microbial diversity compared to the ancestors. The use of these populations for the experiment has been preferred to germ free populations to reduce the experimental burden and prevent the experimental risks and bias of the axenization process (abnormal presence of a contaminating organism, or an increased egg mortality).
For the reciprocal common experiment, including controls, we used around 25 tubes of four and five-days old flies (first and second batch) for each population.

The day before starting the experiment, we visually inspected the number of eggs in each tube and sorted them from the most egg-productive to the least (abondance of eggs visually estimated), in order to distribute flies equally among the cages (see below).

Fruit sampling

Fruits for the reciprocal common environment experiment were collected at two locations in France (Pyrénées-Orientales and Rhône) in June 2023. Each of the two sampling locations included either two (cherry and strawberry) or one (blackberry) cultivation site. As we could not find a farmer growing blackberries in Pyrénées-Orientales, we bought commercial one's that were produced in Corrèze. A few questions were asked to the fruit producers about the production techniques and the fruits' growing conditions since it may impact the composition of the external and internal fruit microbiota. Fruits were brought back to the laboratory and kept overnight at 5°C in a fridge.

Reciprocal common environment experiment

To test for a fruit-specific adaptation driven by the gut microbiota, we used a reciprocal common experiment. It consisted in crossing the nature of the development medium between the hatching phase and the pupariation phase. We put adult females on three types of fruits for the egg laying (step 1, Figure 1), we pulled out the females (step 2), then distributed the larvae in the three types of fruit medium (step 3), and eventually put them in an Eppendorf tube if they turn into a pupa (step 4). Real fruits were used for the egg-laying stage, and lab-made sterile fruit media for the development stage in order to prevent larvae to get microbiota from another source than the real fruit.

To artificially infest fruits with larvae from the two laboratory populations, we first separated females and males on a Petri dish placed on ice (females have a serrated ovipositor and wings without black dots). Prior to any manipulation, flies were anesthetized by putting tubes on ice.
We first placed fruits in embryo collection cages (3.75cm (D) x 5.8cm (H), Genesee Scientific, El Cajon, US). We added 5 or 10 adult females (G0) and allowed them to lay eggs in the fruits. To estimate the natural infestation of the fruits, we also put fruits in cages without any fly (Step 1, Figure 1).
The experiment was conducted in two successive blocks in order to use the fruits from different locations one after the other. After 48 (block 1) or 24 (block 2) hours, we put cages briefly on CO_2 pads to anaesthetize females and removed them with either a sterile paintbrush or dissection forceps (Step 2, Figure 1).

The day following the removal of females, we extracted larvae (G1) from each fruit. Each fruit (stem or calyx removed) was put inside an individual sterile bag[3] (12.5 x 12.5 cm, Whirl-Pak, FisherScientific) and gently pressed with the fingers until the thickness of the formed-puree was uniform. Larvae in the first (L1), or eventually the second or third (L2 or L3) stage, were picked under dissecting microscopes using a small paintbrush previously sterilized in ethanol and transferred in a drop of sterile PBS (1 mL) in a sterile Petri dish.
For each variety of fruit of origin, the same number of larvae was put (using dissection forceps decontaminated with 70% ethanol) in wells of a 96-well plate, each well containing one type of sterile fruit medium (either blackberry, cherry, or strawberry). This step of larval extraction was performed over two days (Step 3, Figure 1).

[3] https://www.fishersci.fr/shop/products/nasco-whirl-pak-easy-to-close-bags-19/11781963

To prevent the exchange of microbes between larvae, they were each isolated in a single well, and the plates were sealed with a sterile and breathable adhesive film (*Aeraseal starter kit* from Sealplate[4], Bernolsheim, France). During the second batch experiment, the adhesive film on plates was renewed twice to prevent the larvae from escaping the well during the pupariation because humidity and manipulation reduced adhesiveness (two pupae in the same well were sometimes observed in both batches).

After three or four days, the presence of pupae in each well was controlled every two days for pupariation of the first batch and every day in the second batch - except on weekends. The 96-well plates were checked with a magnifier under a laminar flow cabinet. To decrease the risk of fly escaping during the control of the pupariation, each pupa was transferred to a sterile 2mL Eppendorf tube with unique barcode using sterile dissection forceps (Step 4, Figure 1). The emergence of adult flies was controlled every day for both batches, except during the weekends.

[4] https://www.ugap.fr/achat-public/aeraseal-starter-kit-sealmate-dispenser-avec-2-rouleaux-de-50-feuilles-sterile_2536084.html

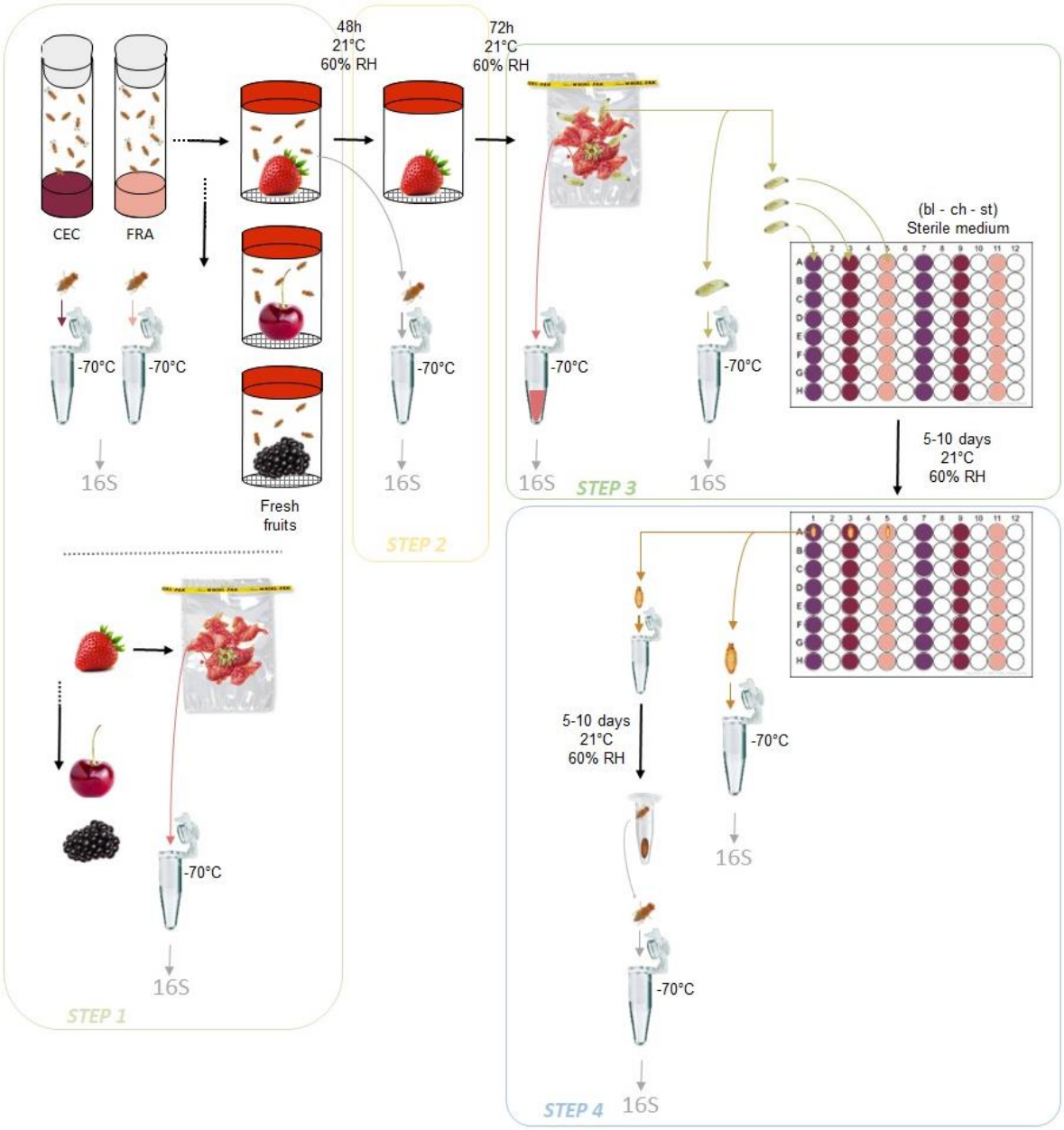

Figure 1. Reciprocal common environment experiment (L. Cesari)
The larvae, hatched from the eggs laid by the female flies in the fruits, are equally transplanted in different fruit media to compare the consequences on larval development. The steps 2-4 are represented only for strawberry, but they were realized also for blackberry and cherry. At each step, individuals are put in Eppendorf tubes in a freezer at -70°C for further genetic analysis (16S sequencing)

Plasticity control

In our reciprocal common environment experiment, the fruit itself rather than its microbial community could affect larval performance. To control for the potential effect of the fruit independently of the microbial community, we performed a control experiment where each fruit of origin in step 1 was replaced with a Petri dish filed with sterile blackberry, cherry, or strawberry medium (10 cages replicates for each fruit medium; Figure 2). The fruit medium of some Petri dishes was not sterile, in which case it was recorded. The rest of the experiment (steps 2 to 4) was identical to the reciprocal common environment experiment described above.

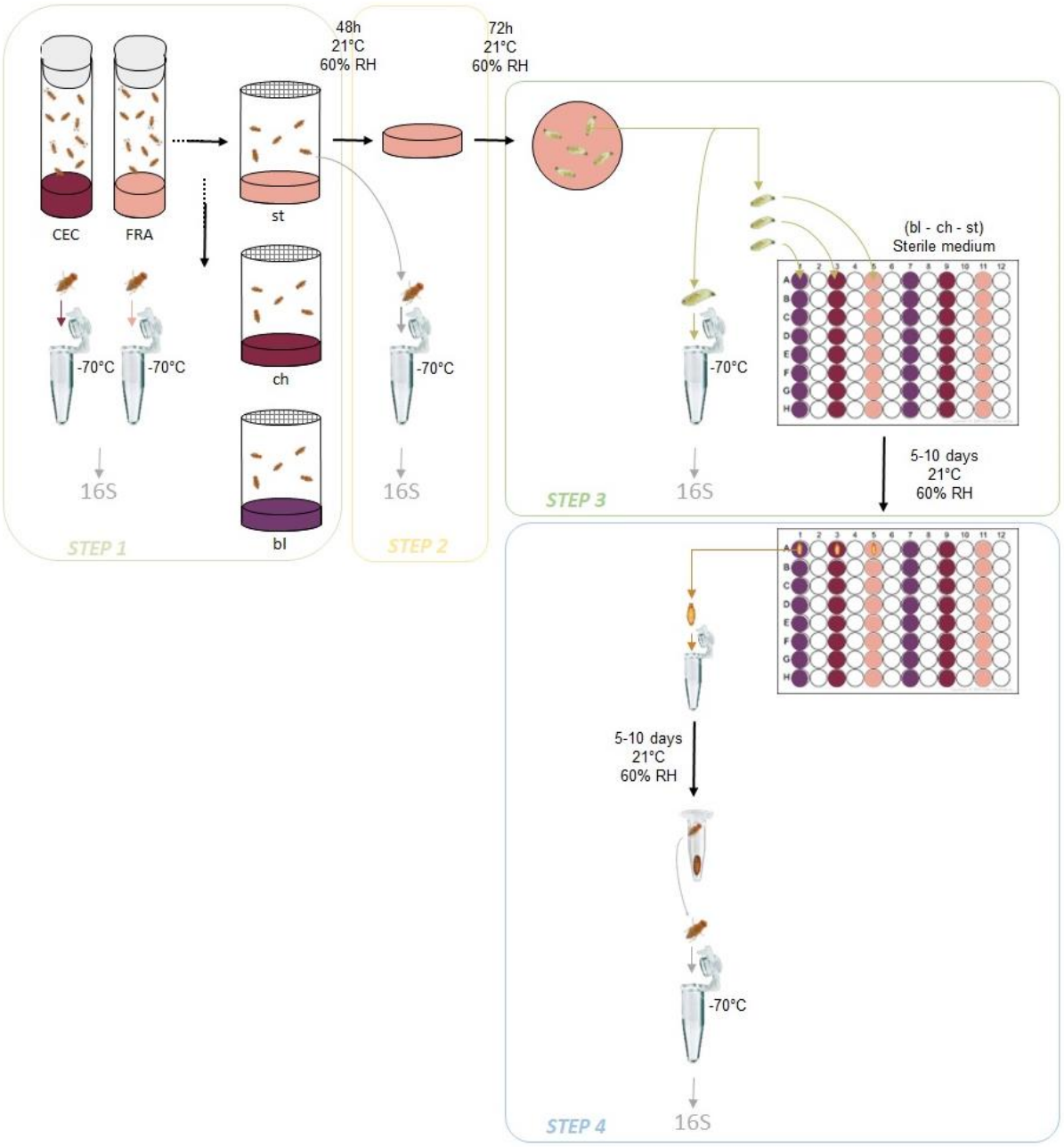

Figure 2. Control of the plasticity (L. Cesari)
The larvae, hatched from the eggs laid by the female flies in the fruit media, are equally transplanted in the three fruits media. The steps 2-4 are only represented for the strawberry medium, but they were realized also for blackberry and cherry media. At each step, individuals are put in Eppendorf tubes in a freezer at -70°C for further genetic analysis (16S sequencing)

Preparation of sterile fruit media

We chose three fruits that can be infested by *D. suzukii* (cherry, blackberry, and strawberry).
To standardize the protocol, we used real fruits for the egg-laying step and prepared artificial fruit media for the larval-development step (see below). The fruit media needed to be sterile in order to prevent the larvae to get another microbial material than the one gained in real fruits (no input of other microorganisms from the development environment), neither alter the effect of the gained gut microbiota on life history traits of the larvae.

The artificial fruit medium was made with frozen purees (*Les Vergers Boiron*, Châteauneuf-sur-Isère, France) were made out of ripe fruits without additional sugar, colouring, or preservatives.
The presence of microorganisms in the purees was tested by plating 50 µL of puree mixed with 50 µL of sterile phosphate-buffered saline (PBS) on lysogeny broth (LB) agar plates, using sterilized glass micro balls. Plates were incubated at 25°C for 5 days, and the presence of microbial colonies was recorded – when present – in a control table (Appendix 1). As the purees were not sterile, I pasteurized them before preparing the medium. I tested different pasteurization procedures (varying temperature, the nature and duration of heat, see Appendix 2 for details). The pasteurization conditions of the taken-on protocol were with direct heating of the stirrer hot plate at 65°C on a magnetic stirrer for 15 minutes. Each fruit medium was prepared by mixing one volume of puree with one volume of boiling agar dissolved in distilled water (2.5% wt/vol), under constant stirring using a magnetic stir bar, on a heating magnetic stirrer.
After each preparation of fruit medium, the presence of microorganisms was controlled by plating 50 µL of fruit medium, with 50 µL of sterile PBS, on LB plates (complete protocol in Appendix 3).
The blackberry and strawberry media used for the second batch were not sterile. I observed a contamination with an approximated concentration of 4-8 colonies/wells (C=0.02-0.04 colony/µL of fruit medium and V_well = 200 µL), but I did not have time to prepare new fruit media before the launch of the experiment.

Statistical analyses

To test whether larvae performed better when transferred to a fruit medium corresponding their fruit of origin, we used two performances proxies: the proportion of larvae that pupated (mean pupariation rate) and the proportion of pupae that emerged as adults (mean emergence rate). All statistical analyses were performed using R statistical software (R-4.3.1 2023). The version control system Git was used for the redaction of the scripts, and the project is hosted on GitHub, Inc. – a platform and cloud-based service for software development and version control using Git – in order to make the results easily reproductible.
I estimated the 95% confidence interval of mean pupariation, and mean emergence rates based on Wilson score formula, using the *BinomCI* function (package *DescTools* version 0.99.49).

To estimate the relative impact of the microbial community on each of the two traits, I first fitted the following model on either pupariation rate or emergence pupariation rate y_{ijkl}, for each batch separately:

$$y_{ijkl} = \text{laboratory\_}population_i + \text{microbial\_community}_j + tested\_fruit_k + laboratory\_population_i{:}microbial\_community_j + laboratory\_population_i{:}tested\_fruit_k + microbial\_community_j{:}tested\_fruit_k + \varepsilon_{ijkl} \quad (1)$$

which includes as fixed effects the *i*th laboratory population of *D. suzukii* (*i* = 1 or 2 for CEC or FRA), the *j*th microbial community depending on the fruit sampled in which eggs were laid (*j* = 1,…,5 for the 5 fruit samples for the first batch; *j* = 1,…,6 for the six fruit samples for the second batch), the *k*th tested fruit medium (*k* = 1,…,3 for blackberry, cherry or strawberry), the interaction between the *i*th laboratory population and the *j*th microbial community, the interaction between the *i*th laboratory population and the *k*th tested fruit, the interaction between the *j*th microbial community and the *k*th tested fruit, and a random error ε.

I tested for the significance of each effect using a F-test (anova function in R). To estimate the relative importance of each effect, I estimated the coefficient of determination of each of the significant terms of the best model.

To test whether each of the two traits had higher values when larvae developed in a fruit medium which corresponded to their fruit of birth (i.e., adaptive phenotypic plasticity), I used the Sympatric-Allopatric (SA) method detailed in Blanquart et al. (2013). To comply with the assumption of a normal distribution, I fitted the following linear model on the *arcsine-square root* transformation of either pupariation rate or emergence pupariation rate, and for each batch separately:

$$S_{ijk} = microbial\_community_i + tested\_fruit_k + fruit\_of\_origin\_of\_microbial\_community_j{:}tested\_fruit_k + SA_{ik} + \varepsilon_{ijk} \quad (2)$$

which includes some of the fixed effects mentioned above, the interaction between the *j*th fruit from which the larva was extracted (j = 1,…,3 for blackberry, cherry or strawberry), a SA factor (which indicates whether the development fruit medium corresponds to the fruit of origin of the microbial community or to an alternative fruits), and a random error ε.
So, a significant difference in the associated *F*-test and a positive estimate of SA provides support in favour of adaptive phenotypic plasticity (a positive estimate of SA indicates better performances in sympatric conditions than in allopatric conditions).

RESULTS

Estimation of the larvae movement within 96-wells plates

Although plates were sealed with adhesive films, and these films renewed for the second batch, we observed some movements of the larvae between their transfer and when the pupariation occurred. In fact, for the first batch 3.15% of the wells (22 wells on the 699 in total) where pupariation occurred contained more than one pupa (i.e., two or three). For the second batch 2.23% of the wells (14 wells on the 627 in total) where pupariation occurred contained more than one pupa.
The slight decrease of this phenomenon in the second batch may be proportional to the pupariation success, which is also lessening in the second batch compared to the first. It might also be due to the renewal of the adhesive film.

Presence of contamination in the 96-wells plates

For both batches, some of the wells filled with sterile fruit media were contaminated by microorganisms. The contamination appeared a few days following the transfer of larvae. For the first batch it represented 7.44% of the wells where larvae were placed, and 77.67% for the second batch. We knew that sterile fruit media used were actually contaminated (cf. Preparation of sterile fruit media in the MATERIAL & METHODS section). The origin of the microorganisms could be the fruit purees used, and/or the gut microbiota carried by the larvae.

Higher pupariation and emergence rates obtained for the first batch

The pupariation success and the emergence success were calculated as the mean of the pupariation success and emergence success of each larva in a taken sample – considered as a Bernoulli variable being equal at 1 if there is a pupa (resp. a fly), and at 0 if there is not. Considering the entire batch

(controls included), we obtained higher pupariation success and emergence success in the first batch (PupSucess_b1 = 0.594 and EmergenceSuccess_b1 = 0.502; Table 1) than in the second (PupSucess_b2 = 0.449 and EmergenceSuccess_b2 = 0.383; Table 1). Besides, all the rates obtained for the first batch are higher than for the second batch, even for more targeted samples, except the emergence rate of the plasticity control which is significatively higher for the second batch (Table 1).

Table 1

	PupSuccess	EmergenceSuccess
Batch01_full	0,594	0,502
Batch01_ReciprocalTransplant	0,721	0,603
Batch01_RT_Allo	0,363	0,303
Batch01_RT_Symp	0,355	0,298
Batch01_PlasticityControl	0,068	0,828
Batch02_full	0,449	0,384
Batch02_ReciprocalTransplant	0,554	0,474
Batch02_RT_Allo	0,273	0,136
Batch02_RT_Symp	0,290	0,232
Batch02_PlasticityControl	0,165	0,252
Batch0102_full	0,516	0,434
Batch0102_RT	0,631	0,512
Batch0102_RT_Allo	0,316	0,245
Batch0102_RT_Symp	0,321	0,260

Testing for the effects of model's factors

We tested the effects of each factor of the model and their interactions (Equation (1)) on the two measured traits, pupariation success and emergence success (Table 2). We calculated the determination coefficient for each significant effect. We found that, for both traits measured, the interaction between microbial community and laboratory population explained most of the variation for batch 2, but not for batch 1 (R^2_Interaction_PupSuccess = 0.512 and R^2_Interaction_EmergenceSuccess = 0.475; Table 2bis in bold).

Table 2

		BATCH 01					BATCH 02				
		Df	Sum_SQ	Mean_Sq	F_value	Pr(>F)	Df	Sum_SQ	Mean_Sq	F_value	Pr(>F)
Pupariation Success	Pop	1	0,003	0,003	0,111	0,747	1	**0,570**	0,570	**45,00**	0,000
	Barcode	4	0,155	0,039	1,591	0,267	5	0,028	0,028	2,194	0,144
	Fruit	2	0,083	0,042	1,710	0,241	2	0,104	0,104	8,203	0,009
	Barcode:Fruit	8	0,167	0,021	0,858	0,583	10	0,010	0,010	0,806	0,631
	Pop:Fruit	2	0,118	0,059	2,431	0,150	2	0,079	0,079	6,264	0,020
	Pop:Barcode	4	0,212	0,053	2,174	0,162	5	0,367	0,367	28,94	0,000
	Residuals	8	0,195	0,024			9	0,013	0,013		
Emergence Success	Pop	1	0,003	0,003	0,454	0,519	1	**0,435**	0,435	**20,81**	0,001
	Barcode	4	0,050	0,013	1,884	0,207	5	0,175	0,035	1,676	0,236
	Fruit	2	0,002	0,001	0,149	0,864	2	0,115	0,057	2,742	0,118
	Barcode:Fruit	8	0,038	0,005	0,715	0,677	10	0,262	0,026	1,252	0,373
	Pop:Fruit	2	0,029	0,015	2,198	0,173	2	0,113	0,056	2,695	0,121
	Pop:Barcode	4	0,024	0,006	0,904	0,505	5	1,163	0,233	11,13	0,001
	Residuals	8	0,053	0,007			9	0,188	0,021		

Table 2bis : determination coefficient of the significative effect for the Batch 02

		R^2
Pupariation Success	Pop	0,182
	Barcode	0,044
	Fruit	0,067
	Pop:Fruit	0,05
	Pop:Barcode	**0,512**
Emergence Success	Pop	0,178
	Barcode	0,071
	Pop:Barcode	**0,454**

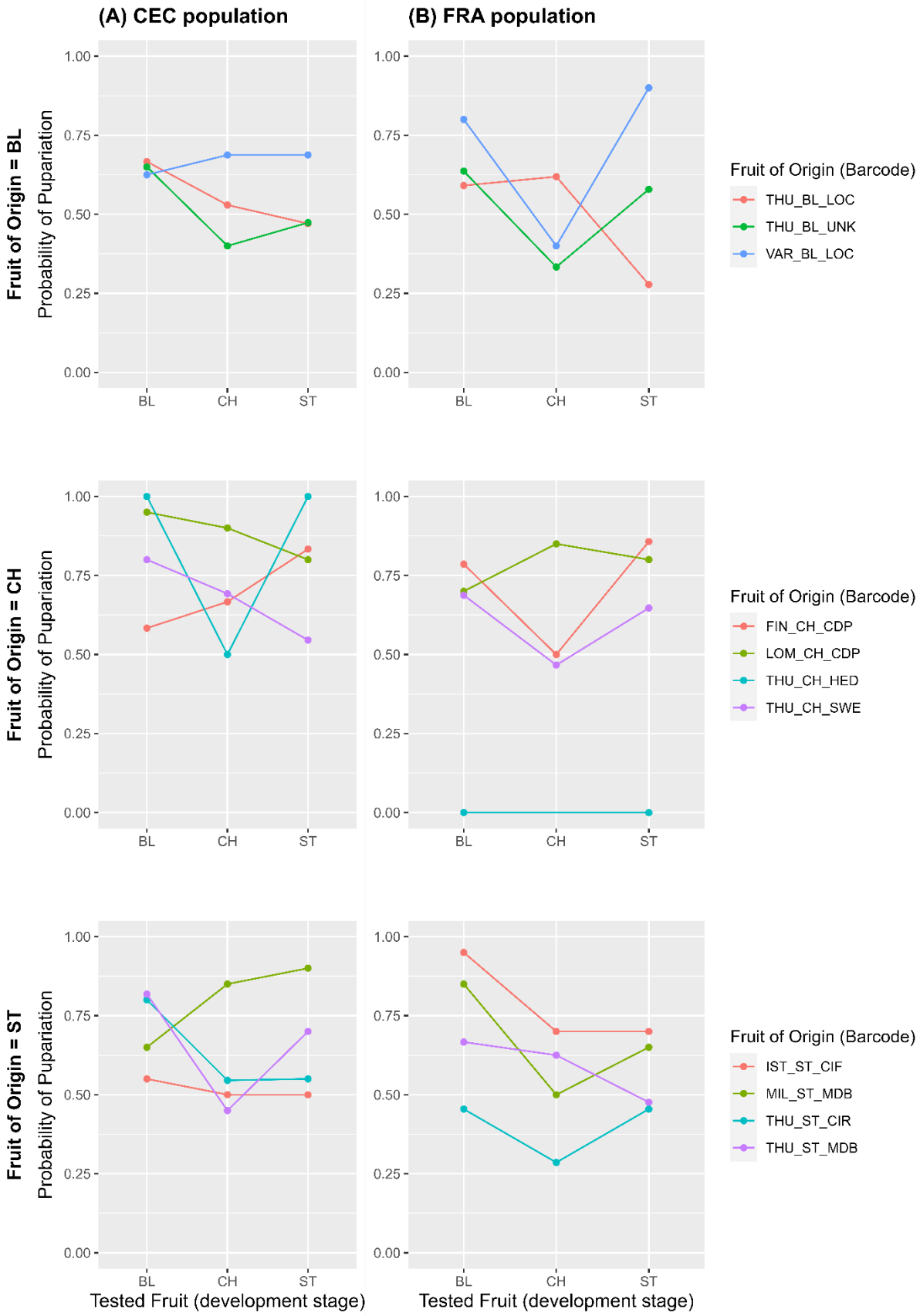

Figure 3. Probability of pupariation of larvae from (A) CEC or (B) FRA laboratory population and which hatched in either blackberry (top), cherry (middle) or strawberry (bottom) fruits with different microbial communities ("Barcode") and developed in three different fruit media (BL: blackberry, CH: cherry, ST: strawberry). The different microbial communities ("Barcode") are listed in Table S2, Appendix 6.

We also plotted the pupariation and emergence successes in function of the microbial community ("Barcode"), considering for each fly population the combined data of the two batches (Figure 3, Figure 4) in order to have a visual pattern of the interaction between the fly population and the microbial community – which significatively explain the variances of the traits. It also permitted to observe the absence of local adaptation, in which case the lines would have been parallels to each other.

The results obtained from the plasticity controls (cf. *Plasticity control* in MATERIAL & METHOD) go along with the effect of the interaction between the population of origin of the flies and the fruit of development observed in the experiment data. For example, the FRA larvae laid on cherry medium has a significant higher pupariation success than the CEC population, whichever the fruit medium of development (Figure S1 and S2 in Appendix 4).

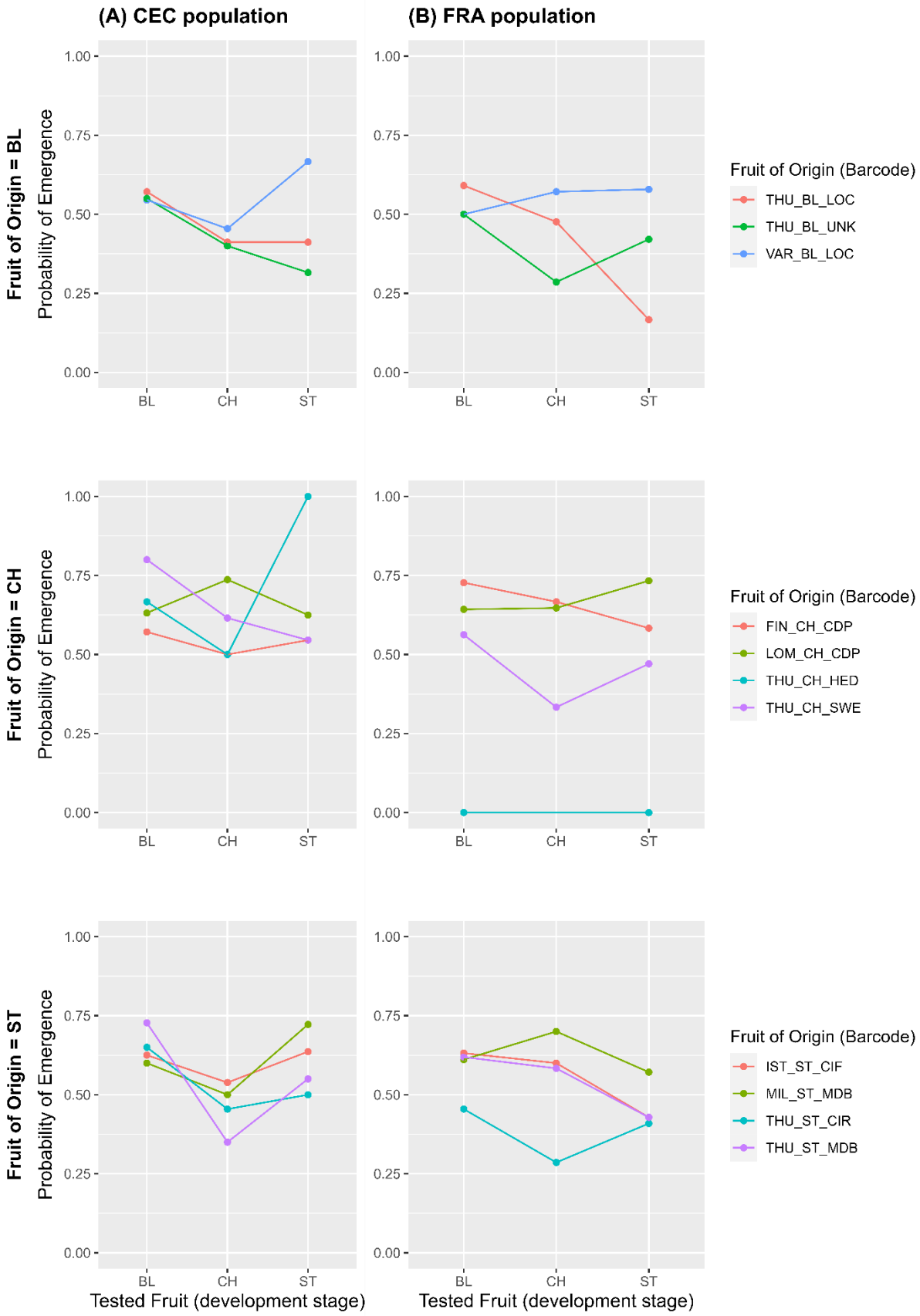

Figure 4. Probability of adult emergence for pupae from (A) CEC or (B) FRA laboratory population and which hatched in either blackberry (top), cherry (middle) or strawberry (bottom) fruits with different microbial communities ("Barcode") and developed in three different fruit media (BL: blackberry, CH: cherry, ST: strawberry). The different microbial communities ("Barcode") are listed in Table S2, Appendix 6.

Testing for local adaptation

The figures 5 and 6 illustrate graphically the pattern observed. The combined data of the two batches are plotted for each fly population (CEC and FRA). The pupariation success (respectively emergence success) in allopatric medium of development – i.e., the larva has been put in a fruit medium corresponding to another type of fruit than its origin one –, is represented in function of the pupariation success (resp. emergence success) in sympatric medium of development -i.e., the larva has been put in the same fruit as the one it is originating from.
If the points are located under the straight line and not above, a pattern of local adaptation is highlighted. Here, whichever the considered the origin of the microbial community, neither the pupariation nor the emergence success is higher in sympatric than in allopatric combinations – the points are not significatively more located under the straight line than above.
When analysing the data from batches 1 and 2 together to test for local adaptation, the ANOVA analysis of pupariation success or emergence success (Equation (2)) revealed that local adaptation does not significatively explain the variance of the interaction between the fruit of origin and the fruit medium (Df_pupSuccess = 1, F_pupSuccess = 0.029, P_pupSuccess = 0.866 and Df_emergenceSuccess = 1, F_emergenceSuccess = 0,176, P_emergenceSuccess = 0,677; Table 3).

Table 3

		BATCH 01 + BATCH 02				
		Df	Sum_SQ	Mean_Sq	F_value	Pr(>F)
Pupariation Success	Barcode	10	0.849	0.08493	1.152	0.346
	Fruit	2	0.205	0.10228	1.387	0.260
	SA	1	0.002	0.00211	0.029	0.866
	Fruit:OriginFruit	3	0.035	0.01175	0.159	0.923
	Residuals	48	3.540	0.07375		
Emergence Success	Barcode	10	0.3732	0.03732	0.567	0.828
	Fruit	2	0.0819	0.04093	0.622	0.543
	SA	1	0.0116	0.01160	0.176	0.677
	Fruit:OriginFruit	3	0.0590	0.01965	0.299	0.826
	Residuals	33	2.1715	0.06580		

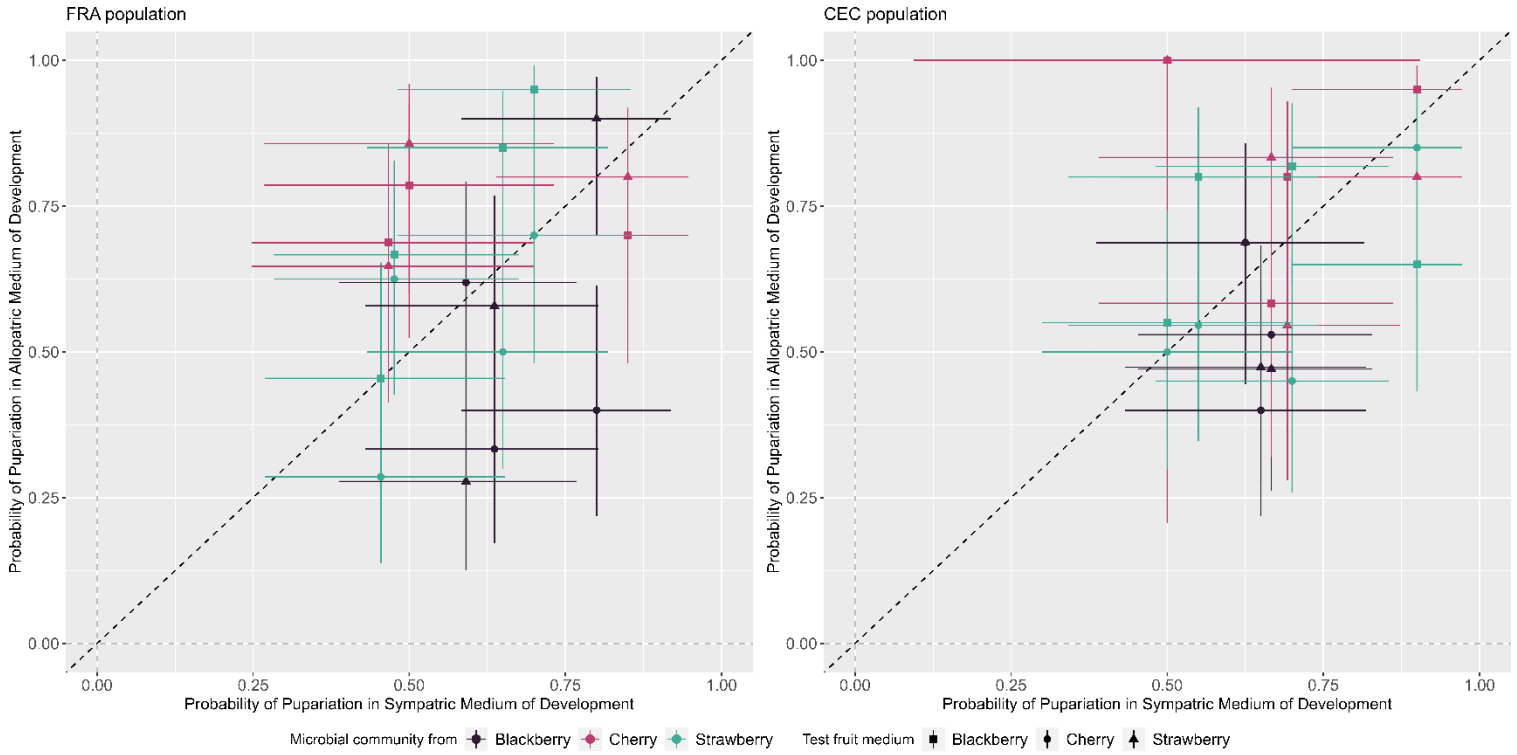

Figure 5. Probability of pupariation of larvae from either the (A) FRA or (B) CEC laboratory population inoculated with different microbial communities ("Microbial community from") and developing in allopatric medium, compared to the probability in sympatric medium of development ("Test fruit medium")

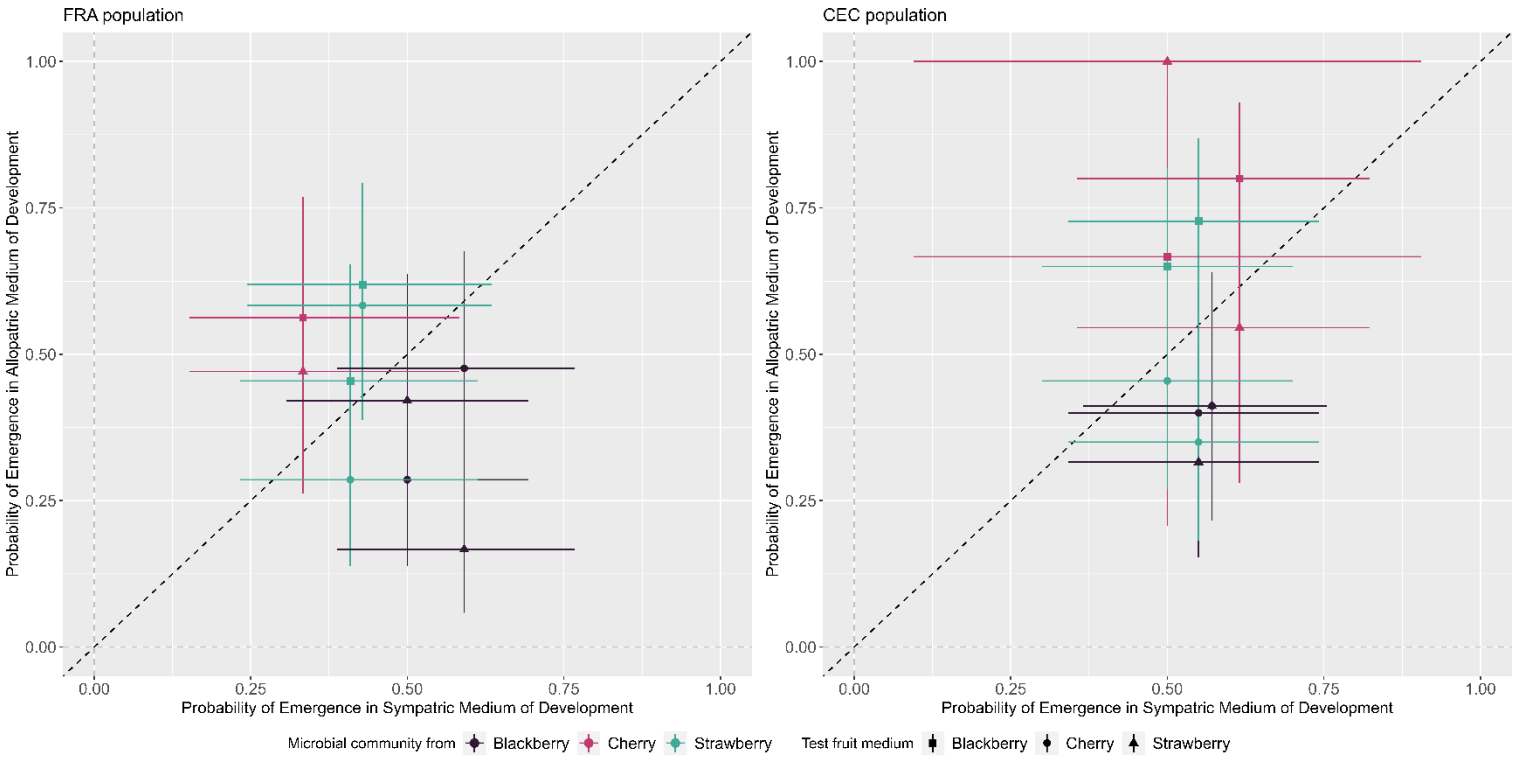

Figure 6. Probability of adult emergence for pupae from either the (A) FRA or (B) CEC laboratory population inoculated with different microbial communities ("Microbial community from") and developing in allopatric medium, compared to the probability in sympatric medium of development ("Test fruit medium")

DISCUSSION

To test for adaptative phenotypic plasticity to specific fruits induced by the gut microbiota, we reciprocally transplanted laboratory populations of *D. suzukii* across three types of fruit media. We replicated this experiment with fruits sampled in two different geographical areas, and measured the pupariation success of the larvae, and the emergence success of the pupae. We found no adaptative phenotypic plasticity for neither pupariation success nor for emergence success.

Relative importance of factors on pupariation and emergence success

Overall, I found no evidence for the existence of fruit-specific adaptation induced by the gut microbiota. Our model included three factors, the population of origin of the flies, the fruit of origin and the tested fruit of development, and their respective interactions. For the pupariation and emergence successes in the first batch, no factor or their interaction has a significant explanatory power (cf. table 1). In particular, there is no interaction between the fruit of origin (which also corresponds to the environmental microbial community encountered by the larva), and the fruit medium in which the larva developed. In addition, as there are no significative interactions, there is no power to test for adaptative phenotypic plasticity using the SA model (equation (2) above). For the second batch, the population of origin ("Pop") explained most of the variance in pupariation and emergence successes (18,2% and 17,8%, table 2bis). Furthermore, the interaction between the population of origin and the fruit of origin (environmental microbial community encountered by the larva; "Pop:Barcode") is significant for the two traits measured (underlined, table 2) and explained 51,2% and 45,4% of the variance in the two traits (table 2bis). This indicates that the effect of the environmental microbial community on the larva varies between our two laboratory populations. The importance of this interaction suggests that the interaction between the fruit of origin (origin of the microbial community) and the fruit medium of development depends on the population of origin (laboratory populations, CEC or FRA). Thus, this interaction decreases the statistical power of the SA model, to detect adaptative phenotypic plasticity as a general phenomenon present whichever the population.

The strong population effect and its interaction with the microbial community encountered by larvae in the real fruit after hatching could have a genetic or a plastic origin. If it is due to a genetic effect, we can hypothesize that in a given environment of development, two strains of *D. suzukii* with different genetic backgrounds origins would react differently to a same microbial community. If it is due to a plastic effect, we can hypothesize that the two fly laboratory populations have different gut microbiota which are transmitted vertically from parents to offspring, and which are responsible for the different larval performances in a given development environment (for instance, pupariation and emergence rates). To discriminate among these two scenarios, I would redo the reciprocal common environment experiment using axenized* larvae from the two populations. It would allow me to test for the sole effect of the gut microbiota that larvae acquired in the fruit after egg hatching on their development.

Hypotheses for the difference of results between the two batches

There is no significant effect for the first batch data and two significant effect and interaction for the second batch data. We can hypothesize that this difference is coming from the different protocols used for the two batches.

Indeed, in the first batch the females were put 48 hours on the fruits to lay their eggs, that is to say 24 hours more than in the second batch (step 2, Figure 1). Consequently, transferred during step 2 were older than in the second batch.
The larval development of *D. suzukii* is separated into three stages based on larval moults (from L1 to L3, cf. Appendix 5) during which the larva may not survive. As for the first batch we mostly put L3 larvae in the 96-well plates, the death rates of L1 and L2 larvae were therefore sidelined, whereas there were included in the second batch since the larvae used were mostly L1 and L2. So, the induced higher mortality rate in the second batch may be at the origin of the difference of effects' influence between batches.
In addition, manipulating larvae in a L3 stage is potentially inducing a higher pupariation rate stimulated by the stress, than larvae manipulated in L1 or L2 stage (Bernays 2009). Those two factors taken together (no small larvae death rate and stress stimulation), induced by the different experiment realizations between batches is a potential explanation of the results differences.

Alternative hypotheses for the absence of adaptative phenotypic plasticity

Based on our results (no adaptative phenotypic plasticity in neither pupariation success nor emergence success) we can make two mutually exclusive hypotheses. First, we can hypothesize that there is no fruit-specific plastic adaptation generated by the gut microbiota.
If this first hypothesis is true, the pattern of local adaptation observed in the study of Olazcuaga et al. (2022) – higher life traits measured for flies developed in the same type of fruit as they originate from than an alternative one – would be due to the specialization of *D. suzukii* to its host fruit. The origin of the pattern would then be genetic, i.e., genetic information is transmitted from parents to offspring and increase egg-to-adult survival in a specific fruit.
Second, the alternative hypothesis would be that there would be a fruit-specific plastic adaptation, but it has not been detected in our experiment. We describe below the possible causes that could be responsible for this.

Factors decreasing the power to detect adaptive phenotypic plasticity

We can make two mutually exclusive sub-hypotheses for a potential lack of detection of fruit-specific plastic adaptation in our experiment. First, we can make the sub-hypothesis that our experiment was not suited to reproduce the results obtained in the study of Olazcuaga et al. (2022). If our experiment was suited to reproduce their finding, we should have observed the same pattern of local adaptation in our plasticity control experiment, where the type of medium for hatching and development is similar (similar microbial communities in both environments). In this case, we should have obtained higher pupariation and emergence rates for larvae transferred in a development medium corresponding to the medium the eggs hatched in (sympatric combinations) than for larvae transferred in a different development medium (allopatric combinations).
One potential explanation for this could be that our fruit medium is slightly different from that of Olazcuaga et al. (2022), potentially resulting in different levels of stress for larvae. Indeed, the fruit media used for the development phase were supplemented with dry yeast in the study of Olazcuaga et al. (2022), but not in ours. It has been shown that depending on the stress level experimented conditioned by the amount of available food, the flies might have a different developmental response (Bing et al. 2018). So, because of a poorer medium of development the flies may not have been in the same developmental conditions in the two experiments, leading to the absence of replication of the local adaptation pattern in our reciprocal common environment experiment.

Second, we can make the sub-hypothesis that some experimental bias prevented the expected course of experimental steps. We describe below some possible experimental bias.

Our experiment aimed at testing whether the microbial community that larvae acquired in real fruit has an effect on the pupariation and the emergence successes. We designed the experiment with the postulate that larvae acquire their gut microbiota as a subsample of the fruit microbiota. As the larvae were not germ free, we can hypothesize that the community present in the fruit may not have been able to colonize the larval gut, which resulted in the absence of microbial transfer (competition among different microbiota can be very important in the larvae gut).

Furthermore, not all the fruit media used for the transfer were sterile. We knew that some sterile fruit media prepared were not sterile thanks to the plating-on-LB test (cf. "*Presence of contamination in the 96-wells plates"* in the results section), and we observed contaminations in the plates during larval development. For the first batch, isolated contaminated wells were contaminated. For the second batch isolated wells and full series of wells were contaminated.
One supposed-fungal species in particular was observed in every type of media (main contaminant), possibly affecting the quality of all media in the same way. However, an additional presence of other isolated supposed-yeast species in contaminated wells was observed. As they were not present in every fruit medium it probably leaded to an imbalanced effect between fruit media on the measured traits.
Besides, negative controls consisting of plates filled with medium but without larvae were realized concomitantly with the plates of the experiment with larvae (second batch only). We observed the growth of the main contaminant a few days after it appeared in the experiment plates, but not the supposed-yeast species. As the later hypothetical yeast species was absent in our control plate grown without larvae, we can hypothesize that; (1) the supposed-fungal species was present in the non-sterile fruit media, and (2) the *ad hoc* supposed-yeast species were present in the non-sterile fruit media and were able to develop thanks to the presence of the larva in the well, or (3) the supposed-yeast species were carried by the larvae (microbiota) and contaminated the medium in which the larvae moved. We may say that contamination was a conflating effect for the realized experiment.

Another potential conflating effect in our experiment lies in the choice of the tested host fruits. The three berries chosen (blackberry, cherry, and strawberry) present a similar adaptative peak for *D. suzukii*, i.e., the death rate of larvae and pupae are similar for these three host fruits. The plastic adaptation to specific fruits governed by the gut microbiota may not have been detected because the gut microbiota taken by the larvae from these three tested fruits are too similar. Using a type of fruit with larvae and pupae death rates significatively different (lower or higher) – implying gut microbiota significatively beneficial or not –, could permit to observe more contrasted results.
Olazcuaga et al. (2019) study on *D. suzukii* oviposition preference highlighted the fact that similar number of eggs were laid on different fruit media (for instance, strawberry, cranberry and apricot; Fig. 1 (Olazcuaga et al. 2019)), but the fly would show different rates of emergence.
For instance, cranberry, fig and apricot have the same number of eggs, but the rate of emergence of cranberry is more than three times higher than in fig and apricot (Olazcuaga et al. 2019). These results suggest that the microbiota carried by these fruits does not have the same importance for larval development, the one in cranberries being potentially more beneficial than those found in figs or apricots. Do the reciprocal common experiment with cranberry, fig, or apricot, and one of the three berries used, may bring more significatively different pupariation and emergence rates.

Plasticity controls highlighted that the microbial community of fruits increases pupariation success

Plasticity controls consisted in putting flies on Petri dishes filed with sterile fruit medium to lay their eggs, instead of real fruits (Figure 2). We observed a lower pupariation success for larvae with the laboratory microbial community than for larvae with the natural microbial community present in the real fruits. Indeed, we observed significantly lower pupariation success in the plasticity control than in the reciprocal common environment experiment. Across laboratory populations and across media of origin, the pupariation success of the plasticity control is less than a third of pupariation success in the reciprocal common environment experiment (Pupariation success of plasticity control of 0,07 and 0,16 in batch 1 and batch 2 respectively, and pupariation success of reciprocal common environment experiment of 0,52 across batch 1 and 2; Table 1).
As the fruit media are supposed to contain the same compounds as the real fruits except for the microbiota, these results suggest that the fruit microbiota does play an important role in the successful development of larvae.

Future statistical analyses

A complementary phase on analysis was planned if I had had enough time. It consisted in measuring and analysing the pupariation and emergence times. For example, flies that had a similar pupariation success could have had different development time. Hence, this analysis would allow to observe whether the gut microbiota has an effect on the duration of the development stages of larvae of *D. suzukii*.
The idea was, to estimate the pupariation and the emergence durations, i.e., the times between the egg-laying date, the pupariation date and the emergence date. As the plates and flies' emergences were not checked on the weekends, the pupariation and emergence dates will be calculated as the mean of the last day without observation and the first day with observation. To analyse those data as continuous variables, a generalized linear model (GLM) will be used.

Another possibility to go deeper in the analysis will be to estimate the effect of the contamination on the success traits measured. For each batch of the experiment, I would calculate the proportion of contaminated wells by category, i.e., depending on the type of fruit media. It will provide information on the effect of contamination on the pupariation or emergence successes, weighed by the number of contaminated samples. This would potentially allow to know the degree to which contamination impacted our experiment (and results).

Future experiments

During the reciprocal common environment experiment, sample of adult flies, real fruits, larvae, pupae, and emerged flies have been conserved at -70°C (every step, Figures 1 & 2). To characterize the microbial community at each step of the experiment, we planned on doing a genetic analysis identify microbes with a 16S gene sequencing. This will permit to characterize and compare the bacterial compositions of the gut microbiota of the adult flies which lay their eggs in the fruits, of the fruits, of the larvae whom eggs have hitched in the fruits, of the pupae and of the flies emerged.
This analysis will permit, among other, to determine if there is a similarity between the microbial composition of the real fruits and of the larvae – which constitute an indicator of a transmission from the fruits to the larvae. Even though, we may consider a beforehand experiment to specifically test for an effective bacterial transmission between the fruits and the larvae, to make sure of the effectiveness of our experiment.

We assume that the taken-on pasteurization protocol did not lead to a proper sterile medium because of a low concentration of the contaminants, not detected when platin media on LB during preliminary experiments. A solution to this problem would be to prepare proper sterile fruit media by realizing an efficient pasteurization of the purees, or by developing a method to sterilize the fruit media after their preparation (UV, …). In order to identify the optimum temperature and duration of pasteurisation, an experiment could be carried out in which the contaminant is inoculated in the medium at a given concentration and then realizing the pasteurization. Analysis of the mortality curves of the contaminant over time and at different temperatures will enable us to identify the pasteurisation conditions that guarantee the absence of contamination.

CONCLUSION

The objective of my internship consisted in testing the effect of the gut microbiota as a plastic evolutionary force for *Drosophila suzukii* – a phytophagous generalist fruit invader originating from Asia. My working hypothesis was that *D. suzukii* larvae acquire their gut microbiota in the fruit they infest, which later allow them to grow better in that specific fruit than in a different fruit, i.e., they develop a fruit-specific plastic adaptation induced by the gut microbiota.

I performed a reciprocal common environment experiment, it consisted in putting fly females on real fruits (equally blackberry, cherry, and strawberry) to let them lay eggs. Then putting larvae hitched from the eggs in different sterile development medium made from fruit purees (blackberry, cherry, and strawberry), and this for each fruit of origin. The media for the development stage was made sterile in order to prevent larvae to get microbiota from another source than the real fruit.

Then, I controlled which day larvae turned into pupae and pupae turned into flies, and I tested whether pupariation and fly emergence rates were higher for the larvae in the same type of fruit they originated from. In particular, I used statistical models to test for adaptative phenotypic plasticity using R-studio. I did not find significant results for neither pupariation success nor for emergence success enabling to pretend for an adaptative phenotypic plasticity induced by the gut microbiota.

From those results two hypothesis appeared, first there is no fruit-specific plastic adaptation generated by the gut microbiota, so our experience was not able to detect significant results; second, alternatively, there is a fruit-specific plastic adaptation generated by the gut microbiota, but our experiment did not permit to detect it. The missuiting of our experiment constitutes a food for thought for the rest of the project on *D. suzukii* gut microbiota. In particular concerning the manipulation of the gut microbiota, it will be important to make sure that the transfer and colonization of the microbiota present in the fruit take place in the flies' gut microbiota. The results of the genetic analysis of the samples taken from our experiment (gene sequencing) will also permit to control the microbial transfer and colonization by identifying the microbial composition and compare it for every step of the experiment.

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APPENDIXES

Appendix 1: control table of the contamination in the fruit purees

Date	ID purée	N° Lot	Test	ProportionInitialePurée (µL/µL)	Dilution	Vétalé_purée+PBS (µL)	Vpurée étalé (µL)	Nbr colonie	[ ] en microbes (nbr cellules viables/µL purée)	Ref photo	
11/04/2023	M1	N222304JA	Dilution	0,16666667	1	100	16,6666667	1	0,06	1104_J3_15; 1104_J13_10	
11/04/2023	M1	N222304JA	Dilution	0,16666667	0,1	100	1,66666667	0	0	1104_J3_15	
11/04/2023	M1	N222304JA	Dilution	0,16666667	0,01	100	0,16666667	0	0	1104_J3_15	
11/04/2023	C1	N121215AA	Dilution	0,16666667	1	100	16,6666667	0		1104_J3_04	
11/04/2023	C1	N121215AA	Dilution	0,16666667	0,1	100	1,66666667	0		1104_J3_04	
11/04/2023	C1	N121215AA	Dilution	0,16666667	0,01	100	0,16666667	0		1104_J3_04	
11/04/2023	F1	N122088BA	Dilution	0,16666667	1	100	16,6666667	6	0,36	1104_J3_10; 1104_J13_11	
11/04/2023	F1	N122088BA	Dilution	0,16666667	0,1	100	1,66666667	0	0	1104_J3_13	
11/04/2023	F1	N122088BA	Dilution	0,16666667	0,01	100	0,16666667	0		1104_J3_9	
11/04/2023	F2	525412F	Dilution	0,16666667	1	100	16,6666667	34	2,04	1104_J3_17; 1104_J13_12	
11/04/2023	F2	525412F	Dilution	0,16666667	0,1	100	1,66666667	2	1,2	1104_J3_11; 1104_J13_13	
11/04/2023	F2	525412F	Dilution	0,16666667	0,01	100	0,16666667	0	0	1104_J3_14	
18/04/2023	M1		Pasteur° 83	0,5	1	100	50	0	0	1804_J2_10	
18/04/2023	C1		Pasteur° 83	0,5	1	100	50	1	0,02	1804_J2_12; 1804_J6_1	
18/04/2023			Pasteur° 83	0,5	1	100	50				
18/04/2023			Pasteur° 83	0,5	1	100	50				
21/04/2023	M1	N122304JA	Pasteur° 63	0,5	1	100	50	0			
21/04/2023	M1	N122304JA	Pasteur° 89	0,5	1	100	50	0			
21/04/2023	C1	N121215AA	Pasteur° 63	0,5	1	100	50	0		2104_J12_6	
21/04/2023	C1	N121215AA	Pasteur° 89	0,5	1	100	50	0		2104_J12_10	
21/04/2023	F1	N122088BA	Pasteur° 63	0,5	1	100	50	5		2104_J12_5; 2104_J18_3	
21/04/2023	F1	N122088BA	Pasteur° 89	0,5	1	100	50	7		2104_J12_8; 2104_J12_11; 2104_J18_2	
21/04/2023	F2	528757F	Pasteur° 63	0,5	1	100	50	17		2104_J12_3; 2104_J18_1	
21/04/2023	F2	528757F	Pasteur° 89	0,5	1	100	50	16		2104_J12_7; 2104_J18_4	
16/05/2023	M1	N122304JA	Pasteur° 60	0,5	1	100	50				
16/05/2023	M1	N122304JA	Pasteur° 60	0,5	1	100	50				
19/05/2023	M1	N122304JA	Pasteur° 70	0,5	1	100	50				
19/05/2023	M1	N122304JA	Pasteur° 70	0,5	1	100	50				purée du 3/05
19/05/2023	F1	N222052AA	Pasteur° 70	0,5	1	100	50				purée du 9/05
19/05/2023	F2	528757F	Pasteur° 70	0,5	1	100	50				purée du 2/05

Appendix 2: table with the different pasteurization protocols and their contamination results (number of colonies for each experimental setup/test)

Date	Type of preparation	Source of heat	Temperature (°C)	Duration (min)	Stirring	Autoclaved beaker	Ice bath	Number of tested purees	Control of contaminations
18/04/2023	sterilized puree	bain-marie	90	2	no	yes	after pasteurization	4 (2 ST, 1 BL, 1 CH)	yes
21/04/2023	sterilized puree	bain-marie	63	3	no	yes	after pasteurization	4 (2 ST, 1 BL, 1 CH)	yes
21/04/2023	sterilized puree	bain-marie	89	1	no	yes	after pasteurization	4 (2 ST, 1 BL, 1 CH)	yes
02/05/2023	fruit medium	bain-marie	63	3	1 min after adding agar solution	yes	no	3 (2 ST, 1 BL)	yes
03/05/2023	fruit medium	bain-marie	63,8	3	1 min after adding agar solution	yes	no		yes
05/05/2023	fruit medium	bain-marie	70	4	1 min after adding agar solution	yes	no	3 (2 ST, 1 BL)	yes
09/05/2023	fruit medium	bain-marie	70	4	1 min after adding agar solution	yes	no	3 (2 ST, 1 BL)	yes
16/05/2023	fruit medium	heating stirrer	66	25	yes	yes	no	1 BL	yes

Date	ID purée	N° Lot	Test	[] purée dans milieu(mL/mL)	Vétalé_milieu+PBS (µL)	Vmilieu étalé (µL)	Vpurée étalé (µL)	Nbr colonie	[ ] en microbes (nbr cellules viables/µL purée)	Ref photo	notes
02/05/2023	M1	N122304JA	Pasteur° 63	0,6857	100	50	34,2857		0	205_J11_3	evaporation dans mélange eau+agar estimée à 31,25%
02/05/2023	F1	N222052AA	Pasteur° 63	0,6857	100	50	34,2857	1	0,029166667	205_J11_2	
02/05/2023	F2	528757F	Pasteur° 63	0,6857	100	50	34,2857	14	0,408333333	205_J11_1; 205_J15_2	
03/05/2023	M1	N122304JA	Pasteur° 63	0,6	100	50	30	0	0		
03/05/2023	F1	N222052AA	Pasteur° 63	0,6	100	50	30	7	0,2333	305_J6_1; 305_J14_1	
03/05/2023	F2	528757F	Pasteur° 63	0,6	100	50	30	19	0,6333	305_J6_2; 305_J14_2	
05/05/2023	M1	N122304JA	Pasteur° 70	0,5613	100	50	28,0645	0	0		
05/05/2023	M1	N122304JA	Pasteur° 70	0,5613	100	50	28,0645	1	0,035632184	505_J4_6	
05/05/2023	F1	N222052AA	Pasteur° 70	0,5374	100	50	26,8707	2	0,07443038	505_J4_3; 505_J12_3	
05/05/2023	F1	N222052AA	Pasteur° 70	0,5374	100	50	26,8707	1	0,03721519	505_J12_2; 505_J12_1	
05/05/2023	F2	528757F	Pasteur° 70	0,5374	100	50	26,8707	20	0,744303797	505_J4_1; 505_J12_5	
05/05/2023	F2	528757F	Pasteur° 70	0,5374	100	50	26,8707	28	1,042025316	505_J4_2; 505_J12_4	
09/05/2023	M1	N122304JA	Pasteur° 70	0,6	100	50	30	2	0,066666667	905_J8_6	
09/05/2023	M1	N122304JA	Pasteur° 70	0,6	100	50	30	1	0,033333333	905_J8_5	
09/05/2023	F1	N222052AA	Pasteur° 70	0,6	100	50	30	0	0	905_J8_4	
09/05/2023	F1	N222052AA	Pasteur° 70	0,6	100	50	30	1	0,033333333	905_J8_2	
09/05/2023	F2	528757F	Pasteur° 70	0,6	100	50	30	13	0,433333333	905_J8_3	purée du 2/05
09/05/2023	F2	528757F	Pasteur° 70	0,6	100	50	30	15	0,5	905_J8_1	purée du 2/05
16/05/2023	F1	N222052AA	Pasteur° 60	0,6	100	50	30				5min
16/05/2023	F1	N222052AA	Pasteur° 60	0,6	100	50	30				10min
16/05/2023	F2	528757F	Pasteur° 60	0,6	100	50	30				5min; purée du 2/05
16/05/2023	F2	528757F	Pasteur° 60	0,6	100	50	30				10min
16/05/2023	F2	528757F	Pasteur° 60	0,6	100	50	30				15min
16/05/2023	F2	528757F	Pasteur° 60	0,6	100	50	30				20min

Appendix 3: protocol of sterile fruit media preparation, including the presence of contamination control

Protocole de pasteurisation des purées par mélange

(avec test conta et coulage)

Matériel
Hotte à flux laminaire (PSM II)
Microonde
Thermomètre
Minuteur
Balance de précision (min 0,001g)
Béchers
Billes de verre stériles
Cuillères
Micropipette 1000/200 µL et cônes
Plaques 96p stériles et/ou petites boîtes de Pétri

Consommables
Aluminium
Purées
Poudre d'Agar
Solution de PBS stérile
Eau osmosée stérile
Boites de Petri avec LB
Film adhésif respirant pour plaque 96p

Recette de base

1 plaque 96 puits = 20 mL de mélange

Mélange	**Pour 20ml**
Fruits	12 ml
Mix Eau Agar à 2,5%	8 ml

Fruits	**100ml**	**12ml**
Fraise	101,0 g	12,12 g
Mûre	111,1 g	13,32 g
Cerise	104,2 g	12,504 g

Agar à 2,5%	**1L**	**8ml**	**8ml + évaporation à 31.25%**
Eau	1L	8 ml	10.5 ml
Agar	25g	0.2 g	0.2 g

Etapes préalables (matin ou la veille)

- Sortir les bacs de purées du congélateur suffisamment en avance pour qu'elles soient malléables
- Autoclaver béchers recouverts d'alu (surface) avec un barreau aimanté placé au fond + cuillères emballées dans de l'alu + plaques 96 puits dans de l'alu + bouteille de PBS + bouteille d'eau osmosée
- Préparer du mélange eau+agar à 2.5% pour chaque purée : peser l'agar grâce à la balance de précision
- Si le milieu est coulé dans des petites boîtes de Pétri, mettre ces dernières et leurs couvercles dans un bain d'alcool à 96% pour la matinée

Etape 1 : pesée des purées

- Allumer et nettoyer la hotte

- Sous la hotte, placer la balance puis ouvrir la boite de purée
- A l'aide d'une cuillère autoclavée, peser le volume de purée désiré dans un bécher autoclavé
- Bien recouvrir chaque bécher en remettant l'alu dessus

Remarque : peser toutes les purées à la suite pour pouvoir enlever la balance de dessous la hotte pour les étapes suivantes

Etape 2 : boite de Petri (test conta facultatif)

- Annoter les boites de Petri (date, fruit, manip)
- Sous la hotte, placer 5 billes de verre stériles par boite de Petri
- Préparer un contrôle positif (50 µL de purée non pasteurisée + 50 µL de PBS stérile) et un contrôle négatif (100 µL de PBS stérile)

Etape 3 : stérilisation de l'eau+agar au micro-onde

- Placer l'agitateur magnétique chauffant sous la hotte
- Le régler au thermostat 1, agitation 8 et placer le premier bécher de purée dessus en le laissant fermé par l'alu
- Mettre la bouteille de mélange eau+agar 30 sec au micro-onde à 750W avec le bouchon déverrouillé d'un quart de tour
- Agiter 10 sec la bouteille pour mélanger son contenu et replacer dans le micro-onde pour un nouveau cycle de 30 sec
- Renouveler l'opération 3 fois (4 x 30 sec au total) en s'assurant que le mélange dans la bouteille soit entré en ébullition

Remarque : prévoir des gants isolants ou feuilles de sopalin pour ne pas se bruler en manipulant la bouteille en sortie de microonde

Etape 4 : pasteurisation du mélange

- Sous la hotte, verser l'eau+agar sorti du microonde dans le bécher de purée placé sur l'agitateur magnétique chauffant
- Nettoyer la sonde du thermomètre à l'alcool à 70% et préparer le minuteur en le réglant à 15min
- Contrôler la température du mélange au thermomètre

Remarque : utiliser la sonde du thermomètre pour bien homogénéiser le mélange si besoin

- Lorsque le mélange atteint 65°C, lancer le minuteur
- Vérifier que le mélange reste bien à 65°C ± 3°C pendant toute la durée de pasteurisation, si besoin enlever le bécher de l'agitateur magnétique et agiter à l'aide de la sonde pour faire diminuer la température
- À 5min de pasteurisation, ajouter 1 mL d'eau osmosée stérile à la micropipette P1000 dans le bécher pour compenser l'évaporation

Etape 5 : étalement sur LB (test conta facultatif)

- Pipeter 50 µL de PBS stérile sur chaque boite de Pétri
- Pipeter 50 µL de mélange purée+agar et étaler sur la boite de Pétri correspondante à l'aide des billes de verre
- Enlever les billes de verre sous la hotte puis placer les boîtes de Pétri à 25°C

Etape 6 : a) coulage du milieu en plaque 96p

- Placer les plaques 96 puits sous la hotte
- Couper l'extrémité du Combitips à l'aide des ciseaux passés à l'alcool à 70%, le placer sur la multipipette
- Régler la multipipette à 200µL par coup
- Prélever le milieu en inclinant le bécher pour ne pas avoir de bulle d'air
- Distribuer tous les X puits (avec X le nombre de fruits utilisés)

Remarque : vider le 1er coup dans le bécher pour s'assurer d'avoir chassé l'air et distribuer rapidement pour limiter la solidification du milieu dans le Combitips

- Laisser refroidir les plaques sous la hotte le temps de pasteuriser les autres purées, faire bien attention à ne pas passer les mains par-dessus les plaques
- À la fin de la pasteurisation, se passer les mains à l'alcool et filmer les plaques à l'adhésif respirant sous la hotte
- Stocker les plaques 96p au frigo dans une boîte de rangement désinfectée

Etape 6 : b) coulage du milieu en petites boîtes de Pétri

- Se passer les mains à l'alcool et sortir les boites de Pétri du bain d'alcool sous la hotte
- Les disposer par lot pour les faire sécher (lot = nombre à couler par type de fruit)
- Couper l'extrémité du Combitips à l'aide des ciseaux passés à l'alcool à 70%, le placer sur la multipipette
- Prélever le milieu en inclinant le bécher pour ne pas avoir de bulle d'air dans le Combitips
- Couler 5 µL dans chaque boîte de Pétri (moitié de Combitips)
- Taper à plat les boîtes de Pétri pour chasser les éventuelles bulles d'air avant que le milieu ne se solidifie
- Laisser refroidir les boîtes sous la hotte le temps de pasteuriser les autres purées, faire bien attention à ne pas passer les mains au-dessus
- À la fin de la pasteurisation, se passer les mains à l'alcool et placer les couvercles sur les boîtes de Pétri sous la hotte
- Les stocker au frigo, à l'envers dans une boîte de rangement désinfectée

Appendix 4 : Figures S1, S2

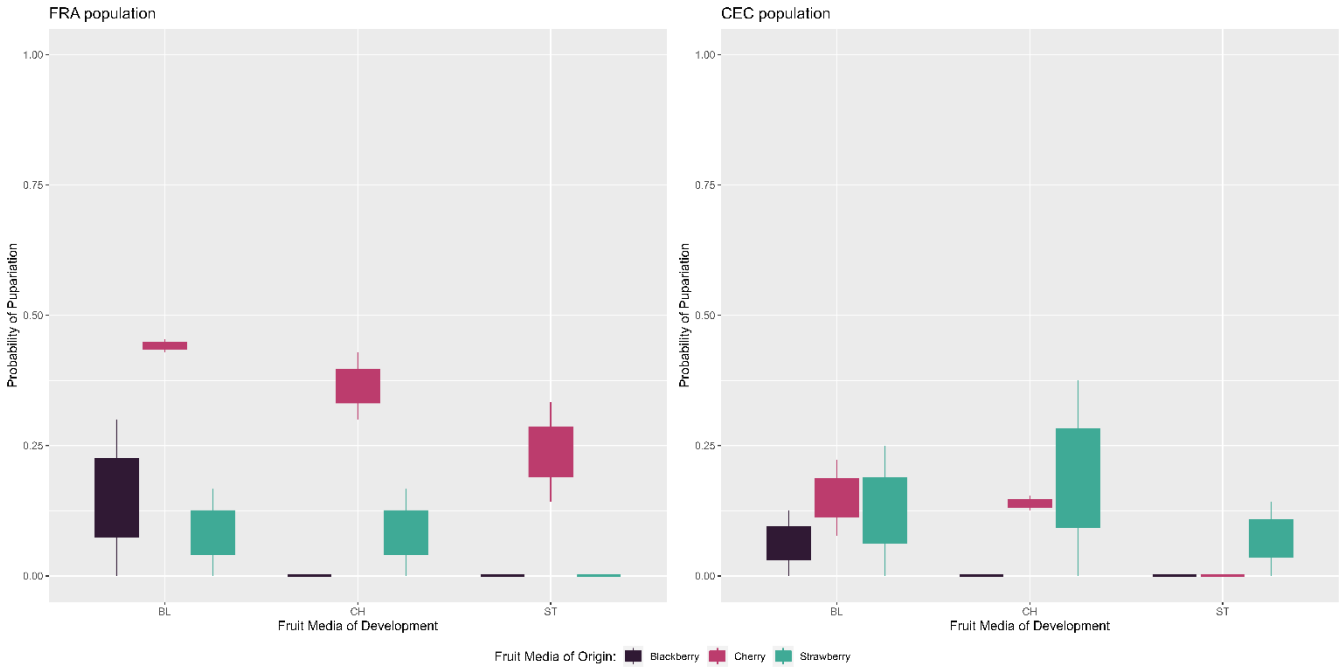


Figure S1. Plasticity control. Probability of pupariation for larvae from FRA or CEC laboratory populations and which hatched in either blackberry, cherry, strawberry sterile fruit media, and developed in the three same fruit media (BL: blackberry, CH: cherry, ST: strawberry).

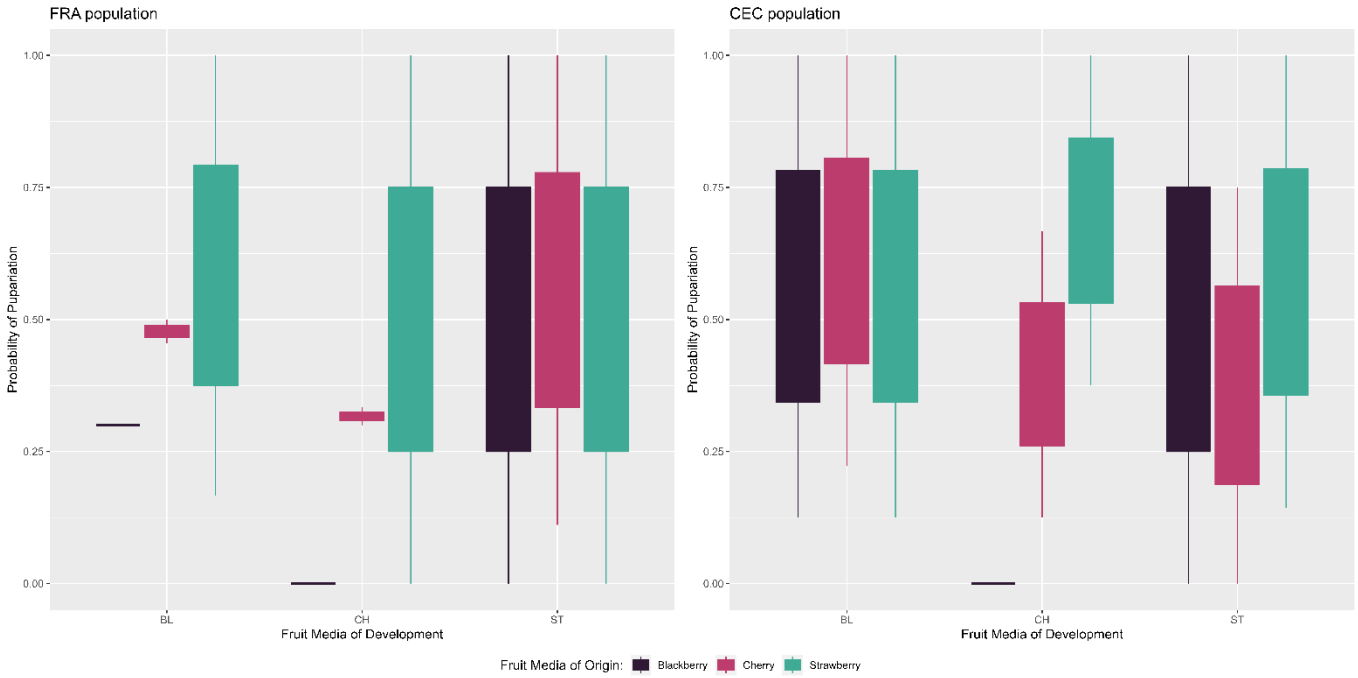
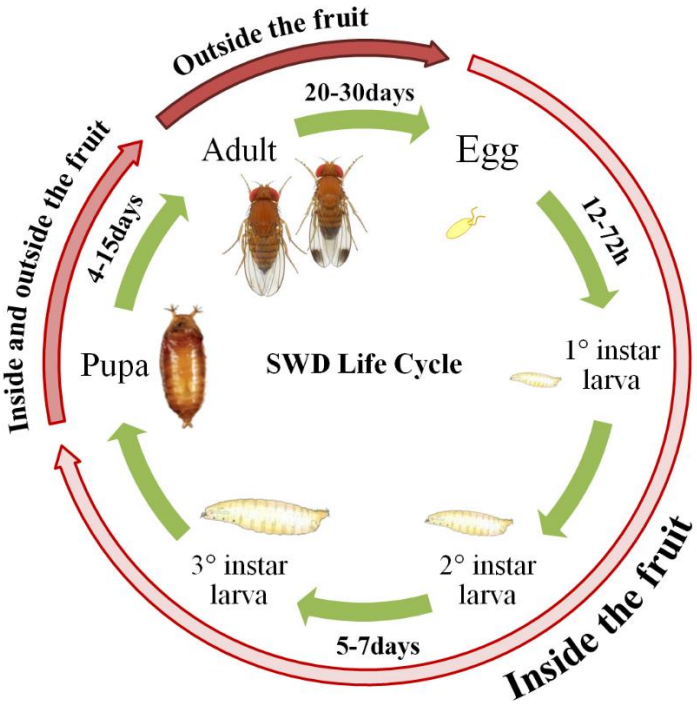


Figure S2. Plasticity control. Probability of emergence for pupae from FRA or CEC laboratory populations and which hatched in either blackberry, cherry, strawberry sterile fruit media, and developed in the three same fruit media (BL: blackberry, CH: cherry, ST: strawberry).

Appendix 5: the life cycle of *D. suzukii*



Source: https://www.agri-mag.com/2022/03/02/drosophila-suzukii/

Appendix 6: Table S2 – Barcode signification

Barcode	***Production Site***	***Type of Fruit***	***Variety***	***Batch***
THU_BL_LOC	Chemin de la Folletière, 69510 Thurins	Blackberry	Locqtay	2
THU_BL_UNK	Grandes Terres, 69510 Thurins	Blackberry	Unknonwn	2
VAR_BL_LOC	Barrière de Bosredon, 19240 Varetz	Blackberry	Lochness	1
FIN_CH_CDP	Lieu dit Cassagne, 66320 Finistret	Cherry	Cœur de Pigeon	1
LOM_CH_CDP	Lloncet, 66500 Los Masos	Cherry	Cœur de Pigeon	1
THU_CH_HED	Grandes Terres, 69510 Thurins	Cherry	Hedelfinger	2

THU_CH_SWE	Chemin de la Folletière, 69510 Thurins	Cherry	Sweetheart	2
IST_ST_CIF	66130 Ille-sur-Têt	Strawberry	Ciflorette	1
MIL_ST_MDB	Mas de Saragosse, Millas	Strawberry	Mara des bois	1
THU_ST_CIR	Grandes Terres, 69510 Thurins	Strawberry	Cirafine	2
THU_ST_MDB	Le Villard, 69510 Thurins	Strawberry	Mara des bois	2

Role of the microbiota in the adaptation of the insect pest *Drosophila suzukii* to different host fruits

Abstract

Organisms become better able to live in their habitat(s) through the evolutionary process of adaptation. Their suited phenotype is the result of natural selection, with genetic shifts through the increase in frequency of selected variants, or of the impact of environment, with plastic shifts through adaptative phenotypic plasticity. Contrary to genetic shifts, plastic shifts provide organisms a fast response to environmental changes. This mechanism plays a key role in species' ecology, in particular among generalist species able to feed in multiple environments. *Drosophila suzukii* is a phytophagous insect pest coming from Asia which invaded Europe, Africa, and America. The aim of my internship was to test for the potential role of the gut microbiota as a plastic evolutionary force for *D. suzukii*. My working hypothesis is that larvae acquire their gut microbiota in the fruit they infest, which later allow them to grow better in that specific fruit than in a different fruit. I performed a reciprocal common environment experiment and tested whether pupariation and fly emergence rates were higher for the larvae in the same type of fruit they originated from than in different types of fruits. I used statistical models to test for adaptative phenotypic plasticity, but I found none for neither pupariation success nor for emergence success. From those results two alternative hypotheses can be drawn, first there is no fruit-specific plastic adaptation generated by the gut microbiota; second, our experiment did not permit to detect the existing fruit-specific plastic adaptation. Future prospects are mainly ruling out potential confounding factors in our experiment and characterizing more specifically the transmission of microbial communities between *D. suzukii* and its host fruit over time, by following the composition of the gut microbiota through experimental steps for instance.

Key words

Adaptative phenotypic plasticity, gut microbiota, reciprocal common environments experiment, *Drosophila suzukii*