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Dissecting yeast-dependent population differentiation and spatial segregation in *Drosophila melanogaster*

Wang, Xiaocui

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Chapter 1
General introduction

Xiaocui Wang

Adaptation to different environments creates and sustains the numerous biological species on our planet. When populations occur in different environments, they may experience divergent selection, which favours different phenotypes in different environments. This may result in divergent adaptation in morphology, physiology and behaviour. Reproductive isolation can emerge in the course of divergent adaptation by various evolutionary mechanisms. One of the main isolating mechanisms - assortative mating (i.e., individuals prefer to mate with mates possessing similar phenotypes) is facilitated when alternative resources are available at different times or locations and mating takes place at those resources (Bush, 1969; Hood et al., 2020). Divergent adaptation and reproductive isolation together mediate the evolution of diverse species (Rundle and Nosil, 2005; Nosil, 2012). Among different environmental factors, food is a major one shaping local adaptation and driving reproductive isolation between populations (Rundle and Nosil, 2005; Nosil, 2012). In this thesis, I specifically focus on exploring how the chemosensory system that is critical for food detection can facilitate food-mediated ecological speciation and experimentally investigating the mechanisms underlying food-mediated population differentiation and assortative mating.

Here, in this introductory chapter, I will give a brief introduction on dietary adaptation and then move to the focus of this thesis. I will also introduce my study system - the interaction between yeast and *Drosophila*- and finish with an overview of my thesis chapters.

Dietary adaptation

Food availability and quality are critical for an individual's growth, survival and reproduction. For instance, variation in nutrients (e.g., protein concentration) of different host plants affects the performance of herbivorous insects including growth and survival (Wetzel et al., 2016). Nutrients contained in food resources can modulate the reproductive behaviour of fruit flies (Gorter et al., 2016). Classic examples of food-mediated divergent adaptation are seen in birds and insects. For instance, feeding on different seeds with distinct sizes promotes divergence in beak size and shape in finches from the Galapagos islands (reviewed in Grant, 2017). Divergent selection from different host plants leads to the diversification of phenotypes and species in herbivorous insects (reviewed in Dres and Mallet, 2002). For instance, different host plants generate changes in the cuticular hydrocarbon (CHC, sex pheromone) phenotypes and assortative mating in two closely related sympatric mustard leaf beetle species, *Phaedon cochleariae* and *P. armoraciae* (Otte et al., 2016). Approximately 3-week earlier ripen apples compared with hawthorn favours earlier (on average about 10 days) eclose time of apple maggot fly, *Rhagoletis pomonella*, and contributes to the allochronic divergence and reproductive isolation between apple race and hawthorn race (Feder et al., 1993). The noni fruit, *Morinda citrifolia*, which can produce toxic hexanoic and octanoic acids engenders *Drosophila sechellia*'s strong tolerance to noni fruits and its isolation from its co-occurring sibling species *Drosophila simulans* (R'Kha et al., 1991; Lavista-Llanos et al., 2014). In this thesis, I specifically focus on dietary adaptation and mainly use examples from herbivorous insects as they have been at the forefront of studies

on dietary adaptation with prominent evidence for dietary adaptation driven by host plants (Dres and Mallet, 2002; Matsubayashi et al., 2010).

Chemosensory system & dietary adaptation - Insects mainly rely on the chemosensory system to find food resources and avoid toxic substances (Smadja and Butlin, 2009). The chemosensory system, including olfactory and gustatory systems, are thus important targets of divergent selection and play an important role in dietary adaptation. It can facilitate dietary adaptation to novel food resources by mediating the detection and identification of these resources through the chemosensory receptors, the genes encoding chemosensory receptors and the odorant-binding proteins (reviewed in Chapter 2 in detail).

Dietary specialization - In nature, food resources are often heterogeneous in terms of chemical composition, spatial distribution, and temporal availability. With heterogeneous food resources, individuals can either maintain high levels of performance over a broad range of diets or specialize on one (Futuyma and Moreno, 1988; Singer, 2008). Maintaining a broad diet can facilitate escaping competition for host resources and provide protection against environmental uncertainty (e.g., temporal/spatial unavailability of food resources or changes in nutritional quality of these resources), but most herbivorous insects are dietary specialists (Hardy et al., 2020). The striking cases of specialized insect host races feeding on different host plants provide strong evidence that dietary specialization on alternative hosts plays an important role in the diversification of herbivorous insects, where reproductive isolation emerges during the process of divergent adaptation to an alternative host (Dres and Mallet, 2002). Yet, how herbivorous insects become specialized rather than remain generalists on different host plants is poorly understood. Interactions between extrinsic environmental conditions (e.g., toxicity of host resources) and individuals' intrinsic abilities (e.g., tolerance to toxicity of host resources) are critical for dietary specialization. For instance, because of tolerance to the toxic hexanoic and octanoic acids produced by the noni fruit – *Morinda citrifolia*, *Drosophila sechellia* can specialize on noni fruit (R'Kha et al., 1991; Legal et al., 1992; Matsuo et al., 2007). Similarly, the monarch butterfly, *Danaus plexippus*, is capable of feeding and living on toxic glycoside-containing milkweeds of the Apocynaceae family because of its resistance to the harmful effects of glycoside (Holzinger et al., 1992; Karageorgi et al., 2019). In addition, dietary specialization often involves a range of traits, including not only digestion and growth, but also reproduction and survival. For instance, the specialization of *D. sechellia* on noni fruit involves its detoxification and digestion and its fertility and offspring development on noni fruits (R'Kha et al., 1991; Legal et al., 1992; Lavista-Llanos et al., 2014). Dietary specialization will be facilitated when these multiple life-history traits depending on the same food source adapt in concert (Rothwell and Holeski, 2020). Therefore, it is important to both examine how extrinsic and intrinsic conditions interact to promote or hinder dietary specialization and systematically measure food-dependent performance for a range of traits.

Food-mediated assortative mating - A critical step of linking specialization to speciation is the occurrence of reduced gene flow between populations specializing on different food

resources. When individuals exploit different food resources and mating takes place on food resources, spatial heterogeneity in food resources can promote segregation and assortative mating between populations. In this scenario, dietary specialization can promote assortative mating and eventually speciation. Food-mediated assortative mating through the spatial coupling of food and mating is one of the most straightforward routes for sympatric speciation (Rice, 1987; Berlocher and Feder, 2002). For example, the apple maggot fly, *Rhagoletis pomonella*, which feeds and mates on hawthorn, formed a specialized apple race with the introduction of apples in North America in the early 1800s and became reproductively isolated from the hawthorn race as it shifted to apple for feeding and mating (Feder et al., 1994; Feder, 1998). Similar processes occurred in the pea aphid (*Acyrtosiphon pisum*) (Caillaud and Via, 2000) and the cactophilic fly (*Drosophila mojavensis*) (Heed, 1978; Fogleman and Danielson, 2001). Though this food-mediated assortative mating has been observed in a range of taxa, the underlying mechanisms allowing the spatial coupling of food and copulation and how this spatial coupling can facilitate food-mediated assortative mating are poorly understood.

Study system

In this thesis, I use *Drosophila* species, particularly *D. melanogaster*, to explore both dietary specialization and food-mediated assortative mating. A growing understanding of behavioural ecology and evolution coincides with unrivalled genetic, genomic and neurobiological data in *Drosophila* in the past few decades. Different *Drosophila* species are attracted to different food resources and there are both dietary generalist species (e.g., *D. simulans*) and specialist species (e.g., *D. sechellia*) within the *Drosophila* subgenus (Figure 1), providing great opportunities to study food-mediated divergent adaptation. These *Drosophila* species are being developed into model organisms for studying dietary specialization, species formation, chemosensation, courtship and mating behaviour. For instance, dietary specialists *D. sechellia*, *D. mojavensis* and *D. erecta* are used to study dietary specialization and speciation, while *D. melanogaster* and *D. simulans* are used to explore the courtship, mating behaviour and the chemosensory pathways involved in food detection and exploitation (Figure 1, reviewed in Anholt, 2019; Anholt, 2020; Chapter 2).

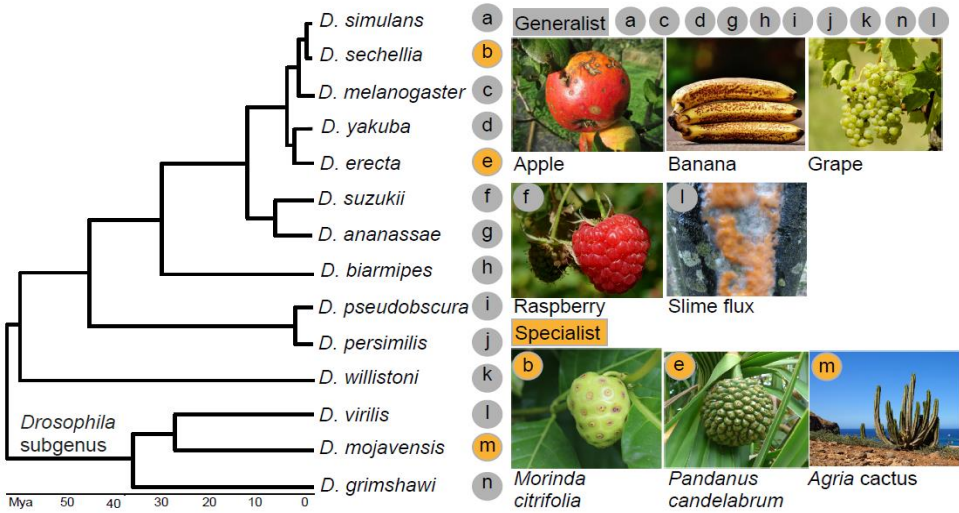


Figure 1: *Drosophila* phylogenetic tree with their associated natural food substrates. Left panel: phylogenetic tree of 14 *Drosophila* species. Right panel: natural substrates exploited by *Drosophila* species. Generalists (marked in grey): *D. simulans* (a), *D. melanogaster* (c), *D. yakuba* (d), *D. ananassae* (g), *D. biarmipes* (h), *D. pseudobscura* (i), *D. persimilis* (j), *D. willistoni* (k) and *D. grimshawi* (n) develop on rotting fruits such as apples, bananas and grapes. *D. suzukii* (f) usually breeds in ripe and soft fruits like raspberries. *D. virilis* (l) feeds on the slime fluxes of toxic trees. Specialists (marked in orange): *D. sechellia* (b) only uses the fruit of *Morinda citrifolia*. *D. erecta* (e) breeds in fruits of various *Pandanus* species such as *Pandanus candelabrum*. *D. mojavensis* (m) has specialized to live on cacti. Food substrate images were taken from <https://pixabay.com/>.

***Drosophila melanogaster* and yeast** – *Drosophila melanogaster* is most often found associated with rotting fruits, flowers, mushrooms and soft rots in nature (Figure 1, Markow and O’Grady 2008) and fermented foods like wine, beer and vinegar in anthropogenic habitats. In fact, it is the yeast, which metabolizes the sugar contained in the above-mentioned substrates, that attracts and supports the development of *D. melanogaster*. Yeast (e.g., baker’s yeast - *Saccharomyces cerevisiae*) is sufficient for *D. melanogaster* attraction and fermenting yeast induced the same fly behaviour as fermenting fruit (Becher et al., 2012). A wide array of yeast species support growth, reproduction and survival in *D. melanogaster*, including the above-mentioned Baker’s yeast, wine-making yeast *Metschnikowia pulcherrima*, dairy yeast *Kluyveromyces lactis*, cactus yeast *Pichia cactophila*, mushroom yeast *Vanrija humicola*, yeast isolated from field-caught flies like *Hanseniaspora uvarum*, *Picha manshurica*, and *Penicillium brevicompactum* and yeast in different forms like live, heated or dried (Anagnostou et al., 2010; Grangeteau et al., 2018; Quan and Eisen, 2018; Murgier et al., 2019; Koerte et al., 2020). The strong dependence of *D. melanogaster* on yeast and the existence

of more than a thousand yeast species offer an opportunity for exploring food-mediated divergent adaptation and is thus the focus of this thesis.

Influence of yeast on *D. melanogaster* life-history traits - Yeast is essential for *D. melanogaster* as it provides direct nutritional benefits. Yeast provides proteins and micronutrients including vitamins, fatty acids and sterols. The dietary supply of sterol from yeast is necessary for the growth of *Drosophila* larvae. In addition to the direct nutritional benefits, yeast is involved in almost all aspects of fly's biology. It modulates *D. melanogaster* performance in several life-history traits, from mating, oviposition to larval development (Figure 2). **Mating** - Mating behaviour is substantially influenced when *D. melanogaster* females and males are on a substrate with yeast. Yeast protein content and its fermentation product acetic acid significantly elevate female receptivity (Gorter et al., 2016). Yeast smell increases male courtship (Grosjean et al., 2011). **Oviposition** - Most *Drosophila* species breeding on fruit resources prefer to deposit eggs on yeast-colonized fruits over fruits dominated by other microorganisms (Oakeshott et al., 1989). Yeast alone can induce flies to oviposit and yeast availability modulates the number of eggs females produce, as *D. melanogaster* ovaries develop more egg chambers on yeast substrates than on sugar medium (Terashima and Bownes, 2004; Becher et al., 2012; Duménil et al., 2016) and the inoculation of substrates with live yeast significantly increases oviposition (Becher et al., 2012). **Larval development** - Yeast is a more complete food compared with bacteria and other fungi that may also have some nutritional value for *D. melanogaster* larvae (Baumberger, 1919). *D. melanogaster* larvae can live on yeast nucleoprotein alone for several days (Baumberger, 1919). Yeast is required for *D. melanogaster* since the larvae develop slowly and die before pupating without yeast (Baumberger, 1919). *D. melanogaster* larvae lack the ability to synthesize sterols, a nutrient essential for structural components of cell membranes and precursors of the moulting hormone ecdysone, but they grow well by utilizing the sterols from yeast (Cooke and Sang, 1970).

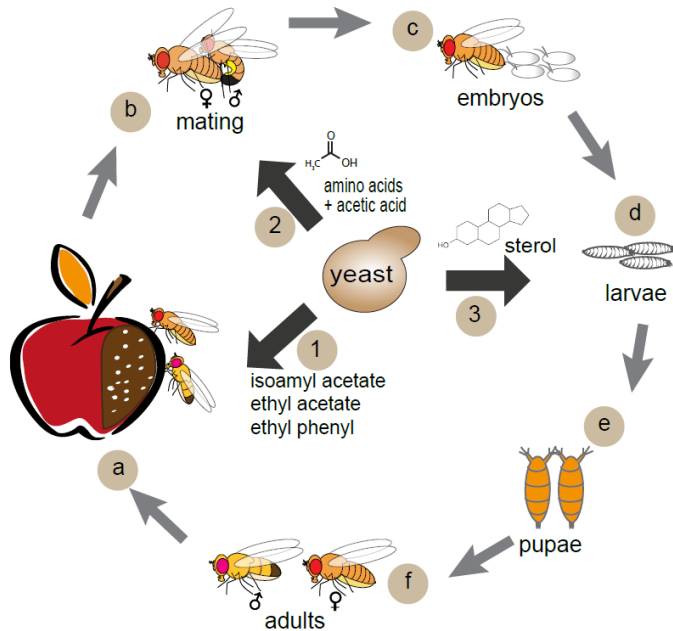


Figure 2: Life cycle of *Drosophila melanogaster* and the chemical cues produced by yeast that are important to the fly's life history. a) Yeast produces chemicals including isoamyl acetate, ethyl acetate and ethyl phenyl (1) (Christiaens et al. 2014) by fermenting fruit; these chemicals attract flies to the fruit. b) In the presence of the amino acids and acetic acids (2), that are generated by yeast fermentation, female flies show increased sexual receptivity and mate more (Gorter et al., 2016). c) After mating, females lay eggs on the fruit with yeast. d) With the sterol (3) from yeast, larvae can successfully develop into pupae (e) and adults (f). Figure adapted from (Billeter and Wolfner, 2018).

Chemosensory responses to yeast in *D. melanogaster* – *D. melanogaster* is tuned to yeast through its chemosensory and physiological responses. Flies detect and discriminate yeast species mainly based on the rich repertoire of chemicals derived from yeast (Becher et al., 2012; Scheidler et al., 2015). Flies display significant attraction to nine yeast species spanning an extensive ecological and phylogenetic range, suggesting that producing chemical cues that attract flies is conserved across yeast clades (Becher et al., 2018). When flies are exposed to traps with several different yeast species, they discriminate between species based on odours (Scheidler et al., 2015). Yeast is transmitted by flies with its attraction and loses its attraction when the alcohol acetyl transferase 1 (*ATF1*) gene contributing to the attraction is deleted (Christiaens et al., 2014).

The chemosensory responses to yeast in *Drosophila* rely on chemoreceptors. In *D. melanogaster*, more than 150 distinct olfactory receptors are distributed in three principal families: the gustatory receptors (GRs), the odorant receptors (ORs), and the ionotropic

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receptors (IRs) (Figure 3 in Chapter 2; (Liang and Luo, 2010)). These chemoreceptors function distinctly to detect yeast and yeast-derived chemicals. Ionotropic odorant receptors, including Ir76b and Ir25a, are required for modulating yeast feeding (Steck et al., 2018). The *Ir75a*-expressing neurons are necessary for the detection of the main component of yeast volatile - acetic acid and the increased female receptivity (Gorter et al., 2016). The *Ir84a*-expressing neurons are activated by yeast volatiles and stimulate male courtship behaviour (Grosjean et al., 2011). Gustatory receptors Gr21a and Gr63a are detectors of the abundant by-product of yeast metabolism carbon dioxide and modulate the attraction or avoidance responses to several odorants in flies (MacWilliam et al., 2018). The gustatory receptor Gr64a is responsible for detecting glycerol, the yeast metabolite under anaerobic conditions (Wisotsky et al., 2011). Altogether, these receptors enable flies to detect, identify and eventually exploit yeast. This implies that understanding chemosensory variation across populations and species exploiting different yeast species will be helpful in understanding yeast-dependent divergent adaptation.

Thesis overview

In this thesis, I aim to understand how heterogeneous food resources can drive population differentiation and assortative mating, using the model organism *D. melanogaster* and its essential food resource – yeast.

Seven questions on the chemical ecology and neurogenetics of resource-mediated speciation

I begin this thesis with a literature review, presenting seven key questions that must be answered in order to understand how the insect chemosensory system can facilitate food-mediated ecological speciation. We focus on the chemosensory system as it plays a fundamental role in food detection, sexual communication and reproductive isolation (**Chapter 2**). We present seven key questions on the chemical ecology and neurogenetics of resource-mediated speciation. We start by identifying which aspects of food resources are heterogeneous in a given environment (Question 1), as this is the starting point of divergent selection. We then discuss which aspects of food exert selection on consumers (Question 2), and explore how consumers detect (Question 3), exploit (Question 4) and adapt to these resources (Question 5) to understand how local adaptation proceeds. Finally, we discuss whether successful exploitation of new food resources is genetically inherited and/or shaped during an individual's lifetime (Question 6) and review the mechanisms that reduce gene flow between individuals that specialized on alternative resources (Question 7). For each of the seven questions, major insights have been generated using various taxa and approaches, documenting the critical resource properties that drive chemosensory adaptations in consumers, the molecular basis of these adaptations and the characterization of the behavioural responses that contribute to reproductive isolation.

How does heterogeneity of food resources affect food-dependent life history traits and drive population differentiation?

Exploitation of food resources is a multifactorial phenotype that involves a range of traits. I conducted behavioural and developmental experiments to measure multiple food-dependent life history traits for exploring how different food resources can drive dietary specialization in consumer populations (**Chapter 3**). I employed seven *D. melanogaster* strains from around the globe and four different yeast species, thereby generating a panel of fly-yeast interactions, and quantified fly responses across multiple yeast-dependent life history traits including feeding, mating, egg-laying, egg development and survival. I found that *D. melanogaster* strains responded differently to different yeast species, indicating distinct interactions between fly strains and yeast species. To explore the occurrence of trade-offs, where better performance on one yeast species comes at the expense of performance on another, as observed in many cases of insect dietary specialization, I measured the correlation of fly performance on alternative yeast species using standardized overall fitness values of fly strains on different yeast species. I found no evidence for trade-offs: fly performance tended to be positively rather than negatively correlated across yeast species. In addition, I also measured the alignment between yeast-dependent life history traits as dietary specialization will be facilitated by the correlated evolution of these traits. I found that the responses to different yeast species were not aligned across traits: different life-history traits were maximized on different yeast species. Finally, we confirmed that *D. melanogaster* is a resource generalist: it can grow, reproduce and survive on all the yeast species we tested. Together, our findings provide a possible explanation for the limited extent of dietary specialization in *D. melanogaster*.

How do heterogeneous food resources become spatially coupled with mates and will this spatial coupling thereby mediate assortative mating?

Food may drive population differentiation through not only its property but also its spatial coupling with mates. When mating takes place on food, assortative mating can arise as a by-product of food choice since individuals that forage on similar food resources are more likely to encounter each other. In this thesis, I also explore how the spatially heterogeneous food resources can be coupled with mating and thereby mediate assortative mating and drive population differentiation (**Chapter 4 & Chapter 5**). For that, I need to monitor both foraging and mating activities of individuals. To track and analyse the two behaviours of *D. melanogaster* in a heterogeneous environment, I first developed a system for video recording, tracking and analysing (**Chapter 4**). I then measured the spatial coupling of foraging and mating and explored the sensory and behavioural mechanisms underlying the co-localization of food and mates (**Chapter 5**). In **Chapter 4**, I present a system of video recording, tracking and data analysis to test a pair of *D. melanogaster* in an arena containing different food patches for long durations (> 12 h) and under different light/dark regimes. With this system, I automatically tracked the positions, the moving distances and the velocities at each position of both males and females as well as the distance between the sexes, courtship behaviour,

and mating location. This system allows exploring the spatial coupling of food and mates, which is the focus of the next chapter. In **Chapter 5**, I tracked the mating location of male-female pairs of wild-type flies (different strains of *D. melanogaster* and strains of other *Drosophila* species including *suzukii*, *simulans* and *yakuba*) in dishes containing distinct food patches. I observed that *D. melanogaster* and its sibling species generally chose to mate on patches containing yeast. *D. melanogaster* was an exception with virgins primarily mating away from yeast, but previously mated females re-mating on yeast. This suggests there is scope for yeast-mediated assortative mating after virginal mating. To explore the sensory mechanism of the spatial coupling of food and mates, I replaced yeast with its main components that modulate mating – the combination of acetic acid and protein. I also generated mutant flies of sex peptide (*SP*) and sex peptide receptor (*SPR*) to explore what causes the behavioural difference between virgin and mated females. We found that mating location preference involved attraction to yeast-derived chemical cues (the combination of acetic acid and protein) and was modulated by the male-derived sex peptide received by females during mating. By taking multiple factors including mating status, light condition and time since first encounter of females and males into consideration, I found that mating location also depended on light conditions and habituation time, with stronger preferences for mating on yeast-containing patches at night than during the day, and an increasing female preference for mating on yeast as time since the first mating elapsed. To further investigate the opportunity for resource-mediated assortative mating between fly populations, I presented several *D. melanogaster* strains with two alternative yeast species simultaneously. We found that *D. melanogaster* pairs preferentially mated on one yeast species over another. Together, our study demonstrates the coupling of food location and mating and suggests a mechanism by which divergence in food preference can directly lead to assortative mating.

In **Chapter 6**, I integrate the findings from all the chapters. I summarize the knowledge gaps we have filled for food-mediated divergent adaptation and discuss the implications of our findings.