Interactions of pollinators and nectar-occupying yeast communities

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SUMMARY

Floral nectar offers a unique niche as habitat for microorganisms. The fluid nectar environment is characterized by a high sugar-concentration, a short flower lifetime and regular nectar depletion and replenishment caused by nectar-foraging pollinators. Microorganisms that live in floral nectar must resist high osmotic pressure, adapt quickly to new environmental conditions, have fast growth rates and manage frequent dispersal to new habitats. Besides generalist bacterial and fungal species that are also known from other sources, there are three yeast species that are almost exclusively found in floral nectar and which are called nectar yeasts: *Metschnikowia reukaufii*, *M. gruessii* and *Candida rancensis*. They can affect their environment by changing nectar chemistry, scent, flower temperature and, as a result, even pollinator behavior. The ecology of nectar yeasts is of interest because of their application as a microbial model system for ecological questions and their potential to change nectar quality and pollinator behavior.

The pollinator has a very special role in the ecology of nectar yeasts as it collects nectar, but also triggers the production of fresh nectar, acts as dispersal pathway for microorganisms and is most likely an unintentional predator when yeasts are swallowed with nectar. The interaction between nectar yeasts and pollinators includes the effects of yeasts on pollinators and vice versa and their dependencies. This thesis focusses on the effects of pollinators on nectar yeasts, mainly by the mechanism of passive dispersal.

To understand local population dynamics of nectar yeasts in single flowers and their stepwise dispersal from flower to pollinator and back to flower, I conducted laboratory experiments with honeybees and artificial flowers and applied an ecological modelling approach (Chapter 1). In the second chapter I describe my investigation of yeast dispersal and metacommunity characteristics under natural conditions in the Uckermark region (Brandenburg, Germany). Finally, I targeted the open question about the overwintering strategy of nectar yeasts in my last chapter, where I tested if yeasts can overwinter with bees (Chapter 3).

In chapter one I attempted to elucidate the conditions under which nectar yeasts reach cell densities with ecological effects. My first finding was that pollination frequency must

be in a beneficial range: too few pollination events reduce yeast inoculation rate and too many strongly reduce local yeast population size. Second, nectar yeasts need traits that ensure at least an intermediate growth rate and an intermediate fraction of yeasts remaining in the flower to compensate for frequent decimation events. Furthermore, I measured that only one out of a thousand cells from a local yeast population reaches the next flower with a pollinator. Those findings suggest that in systems with frequent disturbances, a trait combination of an intermediate persistence ability and intermediate growth rate is optimal rather than maximizing either trait alone.

Investigations of pollinators and flowers from three common tree species in the Uckermark region (Chapter 2) revealed that floral yeast species composition was similar to yeasts dispersed by social insects and dissimilar to yeasts dispersed by solitary insects. Bumblebees and honeybees transported the most nectar-yeasts, wild bees and wasps the most insect-associated yeast species, and sawflies the most transient yeast species. I found strong decrease for yeasts specialized on insects and their nests from pollinator to flower. The results show that the flower visit frequency of the pollinator community plays an important role in determining the species richness and the overall species abundance of the yeasts in flowers, whereas local processes like environmental filtering effects mainly shape the yeast species composition. This approach offers new insights into the role of ecological filters during the dispersal and colonization processes of metacommunities and shows that nectar yeasts offer a unique model system to study the passive dispersal of communities.

In the overwintering experiments with bees I did not find any isolates of specialized nectar yeast species after winter (Chapter 3). However, we found other fungi and yeast species which are known to colonize nectar but are not restricted to the nectar habitat. Some of those occur in very high abundance in floral nectar and are strong competitors with nectar yeasts in floral nectar. Thus, the question of where nectar yeasts overwinter remains open, but we can exclude bees as main vector. Instead, bees offer an overwintering pathway for competitors of nectar yeasts.

This thesis contributes to our fundamental knowledge about nectar yeast ecology and offers insights into several biotic interactions between pollinating insects and yeasts living in floral nectar. I show here that nectar yeasts, from local populations to

metacommunities, are strongly regulated by the density and species composition of the pollinator community.

ZUSAMMENFASSUNG

Blütennektar bietet eine einzigartige Nische als Lebensraum für Mikroorganismen. Flüssiger Nektar ist als Lebensraum charakterisiert durch eine hohe Zuckerkonzentration, eine kurze Lebensdauer der Blüte und regelmäßige Nektarentnahme und -reproduktion, verursacht durch nektarsammelnde Bestäuber. Mikroorganismen, die in Blütennektar leben, müssen hohem osmotischem Druck standhalten, sich schnell an neue Umweltbedingungen anpassen, über eine hohe Wachstumsrate verfügen und den häufigen Transport zu neuen Habitaten sicherstellen. Neben generalistischen Bakterien- und Pilzarten, die auch in anderen Lebensräumen vorkommen, gibt es drei Arten von Hefepilzen, die bisher fast ausschließlich in Blütennektar gefunden wurden und Nektarhefen genannt werden: *Metschnikowia reukaufii, M. gruessii* und *Candida rancensis*. Sie können ihre Umwelt beeinflussen indem sie die chemische Zusammensetzung des Nektars und des Nektarduftes sowie die Blütentemperatur und als Folge davon auch das Verhalten der Bestäuber verändern. Die Ökologie der Nektarhefen ist von Interesse, weil sie als mikrobielles Modellsystem für ökologische Fragestellungen verwendet werden und wegen ihres Potentials die Qualität des Nektars und das Verhalten von Bestäubern zu verändern.

Der Bestäuber spielt eine besondere Rolle in der Ökologie der Nektarhefen, denn er sammelt zwar den Nektar, verursacht dadurch aber auch die Nachproduktion von frischem Nektar, wirkt als Ausbreitungsvektor für Mikroorganismen und ist auch wahrscheinlich unbeabsichtigter Konsument von Hefen durch das Verschlucken von Hefen mit dem Nektar. Die Interaktionen zwischen Nektarhefen und Bestäubern beinhalten die Auswirkungen der Hefen auf die Bestäuber und umgekehrt sowie ihre gegenseitigen Abhängigkeiten. Diese Dissertation befasst sich mit den Auswirkungen der Bestäuber auf Nektarhefen, hauptsächlich durch den Mechanismus der passiven Ausbreitung.

Um lokale Populationsdynamiken von Nektarhefen in einzelnen Blüten und deren schrittweise Verbreitung von der Blüte zum Bestäuber und zurück in die Blüte zu verstehen, nutzte ich ökologische Modellierung sowie Honigbienen und künstliche Blüten für Experimente im Labor (Kapitel 1). Im zweiten Kapitel untersuchte ich die Ausbreitung und die Eigenschaften von Hefe-Metagemeinschaften unter natürlichen Bedingungen in der Uckermark (Brandenburg, Deutschland). Die

Überwinterungsstrategie der Nektarhefen ist noch immer eine ungeklärte Frage. Deshalb prüfte ich im letzten Kapitel, ob Hefen mit Bienen überwintern können (Kapitel 3).

In Kapitel 1 wollte ich wissen unter welchen Bedingungen Nektarhefen Zelldichten mit ökologisch wirksamen Effekten erreichen. Die Antwort besteht aus zwei Teilen. Erstens, die Bestäubungshäufigkeit muss in einem vorteilhaftem Bereich liegen: Zu seltene Bestäubung reduziert die Inokulationsrate der Hefen und zu häufige Bestäubung reduziert die lokale Populationsdichte stark. Zweitens, Nektarhefen benötigen Eigenschaften, die mindestens eine mittlere Wachstumsrate und bei Störung das Zurückbleiben eines mittleren Anteils der Hefepopulation sicherstellen, um häufige Störungen zu kompensieren. Außerdem beobachtete ich, dass nur eine von tausend Hefezellen einer lokalen Population mit einem Bestäuber die nächste Blüte erreicht. Diese Ergebnisse führen zu der Schlussfolgerung, dass es für Lebewesen, die einer häufigen Störungen unterliegen, optimaler ist eine mittlere Wachstumsrate und einen mittleren Störungswiderstand zu entwickeln, anstatt nur eine dieser beiden Eigenschaften allein zu maximieren.

Die Untersuchungen an Bestäubern und Blüten von drei häufigen Baumarten in der Uckermark (Kapitel 2) zeigten, dass eine hohe Ähnlichkeit für soziale Insekten und eine geringe Ähnlichkeit für solitäre Insekten in der Artenzusammensetzung von Hefen, die von Bestäubern transportiert werden und die in Blüten etabliert sind, besteht. Hummeln und Honigbienen transportierten die meisten Nektarhefen, Wildbienen und Wespen die meisten Hefen, die auf Insekten spezialisiert sind, und Schwebfliegen die meisten Hefe-Generalisten. Ich beobachtete einen starken Rückgang von auf Insekten spezialisierte Hefen von der Bestäuber- zur Blütenebene. Die Resultate zeigen, dass die Bestäubungshäufigkeit einen wichtigen Einfluss auf die Vielfalt der Hefearten und deren allgemeine Häufigkeit hat, wobei lokale Prozesse wie das Herausfiltern von Arten durch unpassende Umweltbedingungen hauptsächlich die Zusammensetzung der Hefegesellschaften bestimmen. Die hier verwendete Methode bietet neue Einblicke in die Rolle von ökologischen Filtern während Ausbreitungs- und Kolonisierungsprozessen von Metagesellschaften und zeigt, dass Nektarhefen als Modellsystem für passive Ausbreitung von Gemeinschaften geeignet sind.

Bei Überwinterungsexperimenten mit Bienen fand ich keine einzige Nektarhefenart, die nach dem Winter noch am Leben war (Kapitel 3). Trotzdem fand ich Pilz- und Hefearten, die neben dem Nektar auch in anderen Lebensräumen vorkommen. Einige dieser Arten können sehr hohe Populationsdichten im Nektar ausbilden und sind starke Konkurrenten der Nektarhefen im Blütennektar. Somit konnte zwar die Frage nach der Überwinterungsstrategie der Nektarhefen nicht geklärt, Bienen als Haupt-Überwinterungsmöglichkeit jedoch ausgeschlossen werden. Stattdessen bieten Bienen die Möglichkeit für die Überwinterung von Konkurrenten der Nektarhefen.

Diese Dissertation trägt zum grundlegenden Wissen über die Ökologie von Nektarhefen bei und bietet Einblicke in einige biotische Interaktionen zwischen bestäubenden Insekten und im Nektar lebenden Hefen. Es wurde gezeigt, dass Nektarhefen bereits auf der Ebene der lokalen Population bis hin zu Metagemeinschaften stark von der Dichte und Artenzusammensetzung der Bestäuber reguliert werden.

THESIS OUTLINE

This thesis is a cumulative work, consisting of three chapters that have either been accepted for publication (Chapter 1) or are ready for submission to a peer-reviewed journal (Chapter 2 and 3). The co-authors of the three manuscripts and its contributions are stated below. The general introduction provides background, context and research aims for the works herein and the general discussion section makes conceptual linkages between the results of the studies. The references for each chapter follow that chapter directly. The references cited in the general introduction and discussion sections have been merged into a common reference section at the end of the thesis.

Chapter 1:
Hausmann SL, Tietjen B, Rillig MC. Solving the puzzle of yeast survival in ephemeral nectar systems: exponential growth is not enough.

Author contributions: SLH designed the study, SLH conducted field and laboratory work, all authors contributed to data analysis and interpretation, all authors contributed to the manuscript.

This is a pre-copyedited, author-produced version of an article accepted for publication in *FEMS Microbiology Ecology* following peer review. The version of record
Sebastian L. Hausmann et al. Solving the puzzle of yeast survival in ephemeral nectar systems: exponential growth is not enough. FEMS Microbiology Ecology (2017) 93 (12): fix150
is available online at: https://doi.org/10.1093/femsec/fix150.

Chapter 2:
Hausmann SL, Mittelbach M, Ryo M, Jeltsch F, Rillig MC. Dispersing species pools reveal how mobile links shape metacommunities of nectar yeasts

Author contributions: SLH designed the study and conducted field and laboratory work. SLH, MM and MR analyzed and interpreted the data. All authors contributed to the manuscript.

Chapter 3:
Hausmann SL, Mittelbach M, Rillig MC. Nectar yeast competitors, but not nectar yeasts, overwinter with bees

Author contributions: SLH and MM designed the study and conducted field and laboratory work. SLH and MM analyzed and interpreted the data. All authors contributed to the manuscript.

PUBLICATION LIST

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GENERAL INTRODUCTION

The purpose of the production of floral nectar is to attract pollinators to ensure the transport of pollen between flowers for sexual reproduction of plants (Nicolson, Nepi and Pacini 2007). However, the nectar of flowers is a sugar-rich ephemeral habitat for microorganisms like bacteria and yeasts, too. Nectar yeasts can change nectar chemistry (Herrera, Garcia and Pérez 2008; Vannette, Gauthier and Fukami 2013; Good et al. 2014; Vannette and Fukami 2018), scent (Golonka, Johnson and Hinson 2014; Rering et al. 2017), flower temperature (Herrera and Pozo 2010), pollen transfer rates (Schaeffer and Irwin 2014) and seed production (Herrera, Pozo and Medrano 2013). Bacteria in the nectar mainly decrease the attractiveness of flowers to pollinators while yeasts do not (Vannette, Gauthier and Fukami 2013; Good et al. 2014) or even increase pollinator visitation rate (Herrera, Pozo and Medrano 2013; Schaeffer et al. 2014).

Analysis of the microbial metacommunity of floral nectar identified highly specialized osmotolerant organisms from the ascomycetous family Metschnikowiaceae, mainly *Metschnikowia reukaufii*, *M. gruessii* and *Candida rancensis* (Lachance 2016), which are commonly known as nectar yeasts. In addition to bacteria and nectar yeasts, floral nectar is inhabited by many other osmotolerant transient yeast species, such as generalists or insect-associated species that are primarily associated with other substrates, such as insects or the phylloplane.

Nectar-yeasts as microbial model system

Nectar-borne yeasts have a variety of characteristics that make them suitable as a versatile microbial model system: they depend on pollinators for dispersal (De Vega and Herrera 2012; Schaeffer and Irwin 2014; Pozo et al. 2015), are mainly restricted to the flower habitat (Lievens et al. 2015), have short generation times and low species richness and live in a sugar-rich environment where most microorganisms are filtered out due to high osmotic pressure. The nectar yeast system has been used as model system for many ecological questions dealing with passive dispersal (e.g. Herrera et al. 2010), establishment processes (e.g. Mittelbach et al. 2016a), species-environment interactions (e.g. Rering et al. 2017), competition (e.g. Peay, Belisle and Fukami 2012) and metacommunities (e.g. Vannette and Fukami 2017). Nectar yeasts can be used to test ecological questions under natural conditions with real flowers and pollinators

(e.g. Pozo, Herrera and Alonso 2014; Vannette and Fukami 2017) as well as under laboratory conditions (e.g. Peay, Belisle and Fukami 2012; Tucker and Fukami 2014; Letten et al. 2018) where flowers and pollinators are mimicked by tubes and pipettes (Chappell and Fukami 2018). A major contribution to the field of ecology generated by the use of the nectar yeast model systems is on priority effects. If a species arrives first in a new environment its resource use, growth or even changes in the environment makes it difficult to impossible for later arriving species to establish even if the later arriving species would be more competitive if both arrive at the same time. This exemplary use of nectar yeasts allowed new insights into the strength, persistence (Tucker and Fukami 2014; Toju et al. 2018) and predictability of priority effects (Peay, Belisle and Fukami 2012; Dhami, Hartwig and Fukami 2016) in local communities.

Interactions of pollinators and nectar-occupying yeasts

The nectar yeast metapopulation consists of thousands of flowers representing temporal island habitats that are linked by pollinators. Many studies showed that dispersal of yeasts between flowers is mainly restricted to pollinators as vectors (De Vega and Herrera 2012; Schaeffer and Irwin 2014; Pozo et al. 2015), even if other vectors like wind or precipitation can play a minor role (Pozo, Lachance and Herrera 2012; Schaeffer et al. 2014; Vannette and Fukami 2017). Due to large flight ranges of pollinators, dispersal to a new habitat can occur quickly and over several kilometers. The pollinator serves as the main driver in the system; in addition to dispersing yeasts, it also triggers production of fresh nectar and sustains repeated disturbance events by extracting nectar with a part of the yeast population. Yeasts grow in the nectar of flowers in species-poor communities, typically containing only one and rarely up to 5 different species per flower (own observations from chapter 2). However, the regional species pool can contain many species; up to 26 yeast species were found in nectar of 24 plant species in southern Spain (Pozo et al. 2011).

Although our knowledge on the ecology of nectar yeasts has increased in the last decade, there are still unsolved fundamental questions on population dynamics, the dispersal process and the lifecycle of nectar yeasts. In this thesis, I have targeted open questions that are linked to the interaction of nectar yeasts with pollinators. For simplicity, the term pollinator in this work refers to all insect flower visitors, even if they are not actively pollinating flowers. The interaction between pollinators and nectar yeasts can

be observed from two viewpoints. One perspective is the influence of pollinators on nectar yeasts, which is mainly investigated in this thesis and leads to the conclusion that the system is top-down-controlled by the pollinators as dispersal vectors (Fig. 1). However, nectar yeasts also affect the pollinator community. By changing the nectar chemistry and floral scent, yeasts influence pollinators preferences for flowers which at least partly favor flowers with yeasts (Herrera, Pozo and Medrano 2013; Schaeffer et al. 2014; Fig. 1). Therefore, yeasts can control their dispersal agents bottom-up and thereby secure their dispersal mechanism. This second perspective on the influence of nectar yeasts on pollinators is beyond the scope of the works comprising this thesis but will be addressed in the general discussion.

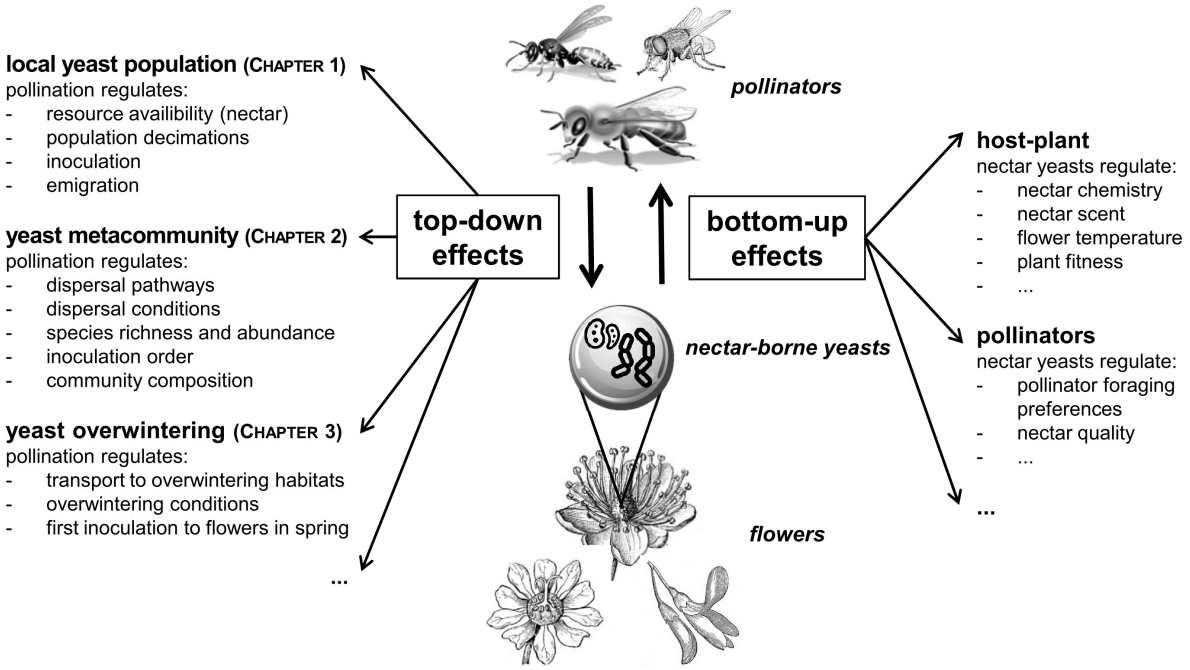

Figure 1: Concept of interactions of pollinators and nectar-occupying yeast communities. Top-down effects of pollinators on nectar yeasts are investigated in the three chapters of this thesis with special focus on local populations (Chapter 1), metacommunities (Chapter 2) and overwintering habitats (Chapter 3).

The effects of pollinators on nectar yeasts are investigated in the three chapters of this thesis. In chapter one I studied population dynamics of nectar yeast in single flowers under different pollination scenarios, which is necessary to predict when and under what conditions yeasts start to have ecological effects on their environment. Then I studied how dispersal affects metacommunities on the regional scale under natural conditions in chapter two. In the last chapter I tested a hypothesis by Brysch-Herzberg

(2004) suggesting that nectar yeasts overwinter with bees, which to date had never been tested. In the next paragraphs the three chapters will be described in more detail.

Background on local population dynamics (Chapter 1)

In the first chapter, I investigate population dynamics in single flowers under different pollination frequencies to predict ecological effects. A new nectar yeast population starts with the flower visit of a pollinator carrying yeast cells. The chance of successful inoculation and the number of cells dispersed to the flower depend on the number of cells the pollinator took up from the source flower, the individual foraging behavior (movement) and physiological traits (e.g. size, shape and surface structure) of the pollinator and the yeasts cells. Most cells removed from the floral nectar were swallowed by the pollinator and just the ones that stick to the pollinator surface have the chance to be dispersed to new flowers. Since the nectar is partly or totally depleted by the pollinator, growth of yeasts can be slowed down until the flower starts producing fresh nectar. If the environment in the flower is suitable, the yeasts need a few hours to adjust to the new conditions and eventually compete for resources. Population growth can be interrupted when the next pollinator visits the flower. By then, new yeast cells have the chance to be inoculated to the flower. The growth, decimation and inoculation cycle caused by pollination events repeats until the flower is fully pollinated and nectar production stops.

From previous literature it is known that ecological effects of nectar yeasts on pollinator behavior (Herrera, Garcia and Pérez 2008; Herrera, Pozo and Medrano 2013; Schaeffer et al. 2014), flower temperature (Herrera and Pozo 2010) or plant fitness (Herrera, Pozo and Medrano 2013; Schaeffer and Irwin 2014) are only detectable if the yeast population reaches a density higher than $5x10^4$ cells μl^{-1}. To reach that density yeasts need some days to grow, but during this time the population can be disturbed several times by pollination events when nectar and a part of the yeast community is depleted by pollinators. I used a system with honeybees and artificial flowers to assess the remaining fraction of the yeast population in the flower after nectar foraging of a pollinator and measured the number of yeast cells that is dispersed by a pollinator from one to the next flower. With these data and an ecological modelling approach, I simulated nectar yeast populations under different scenarios for dispersal, growth rates, adaption to dispersal and pollination (disturbance) frequencies to find out under which

conditions nectar yeasts can compensate for the population loss during pollination and reach ecological relevant densities after inoculation.

The following questions are addressed in the first chapter:

- What proportion of the yeast population remains in the flower after nectar foraging by a pollinator?
- How many yeast cells are transported by a pollinator from on flower to the next?
- Under what conditions do nectar yeast reach densities with ecological effects?

Background on metacommunity dispersal (Chapter 2)

After gaining insights into population dynamics in single flowers, I wanted to investigate the processes between several flowers forming nectar yeast metacommunities and how they are dispersed (Chapter 2). The nectar yeast system offers the opportunity to investigate yeast metacommunities during dispersal on the pollinator mouthparts and after establishment in the flowers. Previous studies that identified yeasts attached to pollinator bodies only investigated small parts of the pollinator guild (Lachance et al. 2001; Rosa et al. 2003; Brysch-Herzberg 2004; Herrera et al. 2010; Mittelbach et al. 2016). However, analyzing the whole pollinator spectrum seems necessary to gain an improved understanding of frequently disturbed metacommunity structure, diversity and dispersal of yeast. I sampled flowers and pollinators of three common tree species, identified attached yeast species and observed flower visits of different pollinator groups. Thus, I could compare how the yeast community change from pollinator to flower level under different pollinator assemblages and dispersal frequencies in the field.

The following questions are addressed in the second chapter:

- How do yeast communities isolated from floral nectar and isolated from the pollinating mobile linker community differ?
- How does pollination frequency affect nectar yeast communities?
- Do specific pollinator groups transport specific nectar yeast species?

Background on nectar yeast overwintering (Chapter 3)

The third chapter addresses a more general question on the nectar yeast lifecycle. Even if the ecology of nectar yeast communities during vegetation period is studied extensively, it remains unclear how and where they survive the winter period and become inoculated into the nectar of flowers in spring again. A common hypothesis is that nectar yeasts endure the winter with pollinating insects (Brysch-Herzberg 2004), either on their body surface or in the stored food. However, this hypothesis has not yet been tested. Overwintering strategies that do not involve insects seem to be unlikely –an introduction and overwintering of nectar yeasts in buds has not been shown to date and the fact that nectar yeasts have never been observed as endophytes in culture-based studies (Fonseca and Inácio 2006), reduces the likelihood that they establish from floral tissues. As far as soil is concerned, several studies on yeasts in soil never found these species in different land use types in Germany (Yurkov, Kemler and Begerow 2012) and Slovakia (Sláviková and Vadkertiová 2000, 2002, 2003). Bees as the main pollinators in Europe (Williams, Corbet and Osborne 1991) disperse a large proportion of nectar yeast populations. Thus, it is reasonable to hypothesize that yeasts use pollinators, their nesting site or food reserves as overwintering sites, as proposed by Brysch-Herzberg (2004). Therefore, bees and their food reserves were analyzed for nectar yeasts after hibernation and before pollinators leave their nests in spring. Three bee groups with different social organizations and hibernation strategies were studied: honeybees, bumblebees and wood-nesting solitary bees.

The following question is addressed in the third chapter:

- Do nectar yeasts overwinter with bees or in bee nests?

Integration into general ecological research

Although this research uses nectar-borne yeasts as study organisms, the questions addressed in the first two chapters can be linked with general ecological processes. In chapter one, nectar yeast population dynamics serve as a model for systems where local populations need to persist during disturbance or dispersal events as well as compensate for periods of high mortality or emigration. These conditions can be found in a variety of systems, including stream drift, floods, wind drift, in the microbial communities of water tanks of Bromeliads and in puddles or ponds. In chapter two, I

analyze the composition of migrating species pools from different passive dispersal pathways (here: different insect pollinator groups) and compare the species composition with the established metacommunity in the local (flower) habitat. These multifaceted investigations of a (pollinator-) community that act as a mobile linking system for another community can offer a deeper insight into the dynamics of frequently disturbed metacommunity to identify the role of regional and local processes in metacommunity formation. The third chapter tests a longstanding hypothesis on nectar yeast overwintering. This specific question on the nectar yeast lifecycle cannot be enlarged to a broader ecological theory. However, it is fundamental to understand the whole lifecycle of nectar yeasts in order to be able to protect them.

All chapters are based on the mechanism of passive dispersal by flower-visiting insects and investigate the consequences of this activity for populations in the local flower habitat (Chapter 1), for metacommunity formation (Chapter 2) and for the lifecycle during winter time (Chapter 3, Fig. 1). With this research I aim to contribute to our fundamental knowledge on the ecology of nectar yeasts, especially on their interactions with flower-visiting insects.

1 CHAPTER 1: SOLVING THE PUZZLE OF YEAST SURVIVAL IN EPHEMERAL NECTAR SYSTEMS: EXPONENTIAL GROWTH IS NOT ENOUGH

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1.1 Abstract

Flower nectar is a sugar-rich ephemeral habitat for microorganisms. Nectar-borne yeasts are part of the microbial community and can affect pollination by changing nectar chemistry, attractiveness to pollinators or flower temperature if yeast population densities are high. Pollinators act as dispersal agents in this system; however, pollination events lead potentially to shrinking nectar yeast populations. We here examine how sufficiently high cell densities of nectar yeast can develop in a flower. In laboratory experiments, we determined the remaining fraction of nectar yeast cells after nectar removal, and used honeybees to determine the number of transmitted yeast cells from one flower to the next. The results of these experiments directly fed into a simulation model providing an insight into movement and colonization ecology of nectar yeasts. We found that cell densities only reached an ecologically relevant size for an intermediate pollination probability. Too few pollination events reduce yeast inoculation rate and too many reduce yeast population size strongly. In addition, nectar yeasts need a trait combination of at least an intermediate growth rate and an intermediate remaining fraction to compensate for highly frequent decimations. Our results can be used to predict nectar yeast dispersal, growth and consequently their ecological effects.

1.2 Introduction

The nectar of flowers is a sugar-rich ephemeral habitat for microorganisms such as bacteria and yeasts. Nectar microorganisms are known to affect plant–pollinator interactions. Bacteria in the nectar mainly decrease the attractiveness of flowers to pollinators, while yeasts do not (Vannette, Gauthier and Fukami 2013; Good et al.2014) or even increase pollinator visitation rate (Herrera, Pozo and Medrano 2013; Schaeffer et al.2014). Here we focus on the ecological effects and movement ecology of nectar

yeasts and not bacteria because nectar yeasts depend on pollinators for dispersal (De Vega and Herrera 2012; Schaeffer and Irwin 2014; Pozo et al.2015), are mainly restricted to the flower habitat (Lievens et al.2015) and strengthen the plant–pollinator interaction. Nectar yeasts can also change nectar chemistry (Herrera, Garcia and Pérez 2008; Vannette, Gauthier and Fukami 2013; Good et al.2014), flower temperature (Herrera and Pozo 2010), pollen transfer rates (Schaeffer and Irwin 2014) and seed production (Herrera, Pozo and Medrano 2013). The size of effect is very likely to be dependent on yeast cell density, and empirical observations suggest that effects of nectar yeasts on pollinators and plants are likely to occur only if yeast densities are higher than 5×10^4 cells μl^{-1}. When yeast cell density was lower than 10^4 cells μl^{-1}, there was no difference in nectar attractiveness to pollinators, and only small changes in nectar chemistry were detected (Vannette, Gauthier and Fukami 2013; Good et al.2014). Changes in nectar sugar concentration and composition were observed by Herrera, Garcia and Pérez (2008) when nectar yeasts reach densities between 10^4 and 10^5 cells μl^{-1}. For these densities, nectar became more attractive to pollinators than nectar without yeasts (5×10^4 cells μl^{-1}: Schaeffer et al.2014; 10^5 cells μl^{-1}: Herrera, Pozo and Medrano 2013). Conflicting results were found for plant fitness: both a positive effect on pollen transfer rates (Schaeffer and Irwin 2014) and a negative effect on produced seed mass (Herrera, Pozo and Medrano 2013) were found for cell densities above 10^5 cells μl^{-1}. Cell densities higher than 5×10^4 cells μl^{-1} were found to warm the flowers of a winter-blooming plant (Herrera and Pozo 2010), representing a temperature reward for the pollinator.

The nectar yeast metapopulation consists of thousands of flowers representing temporal island habitats that are linked by pollinators. The pollinator is a key element in this system with a complex set of roles including dispersal agent, competitor for nectar resources and incidental consumer. A new nectar yeast population starts with the flower visit of a pollinator carrying yeast cells (Fig. 2). The chance of successful inoculation and the number of cells dispersed to the flower depend on the number of cells the pollinator took up from the source flower. Since the nectar is partly or totally depleted by the pollinator, growth of yeasts can slow down until the flower starts producing fresh nectar. If the environment is suitable, the yeasts need a few hours to adjust to the new conditions and eventually compete for resources. Population growth can be interrupted when the next pollinator visits the flower. The pollinator takes up nectar and any yeast

in it. Growth of the remaining population is again slowed down until nectar is replenished. Most cells taken will be swallowed by the pollinator (mortality during emigration) and just a small proportion has the chance to be dispersed to new flowers. The growth and decimation cycle caused by pollination events repeats until the flower is fully pollinated and nectar production stops.

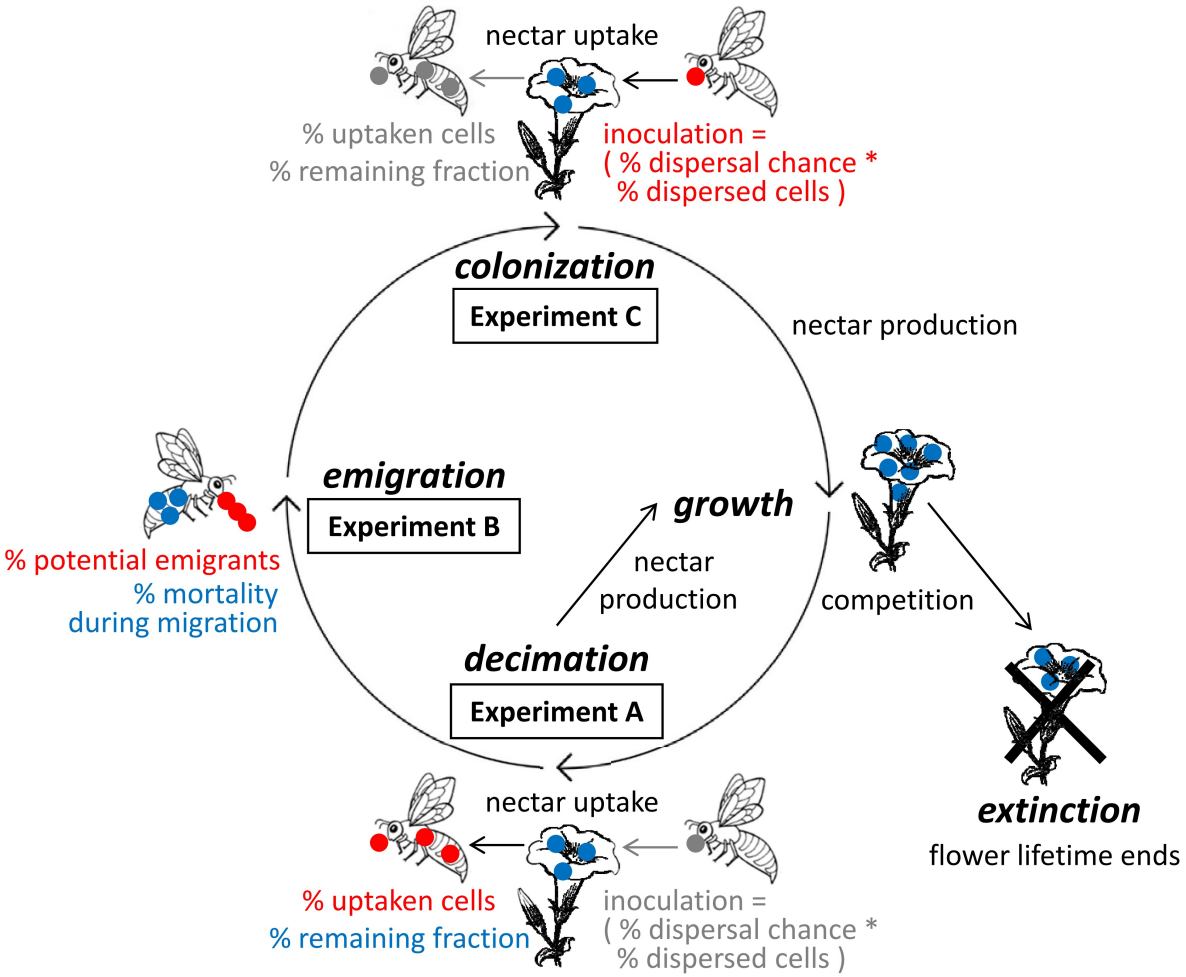


Figure 2: Simplified depiction of the movement ecology of nectar yeasts with a focus on dispersal by pollinators. The black bold italic terms are main steps in the lifecycle, the terms in black regular font are processes, and the variables in red and blue were measured in our experiments. Terms in frames show which lab experiment represents which dispersal step of nectar yeasts. Colonization and decimation can happen during the same pollination event if yeast population is already established.

Microorganisms are not only transferred between flowers by pollinating insects but also by other nectar consumers, including ants (De Vega and Herrera 2012) and birds (Mittelbach et al.2015), or by abiotic factors like wind or precipitation (Samuni-Blank et al.2014). For nectar yeasts, many studies showed no flower inoculation if pollinators are excluded by mesh bags (De Vega and Herrera 2012; Schaeffer and Irwin 2014; Pozo et al.2015). In contrast, some studies show that excluding pollination by mesh bags

reduces but cannot totally prevent inoculation by nectar yeasts (Pozo, Lachance and Herrera 2012; Schaeffer et al.2014; Vannette and Fukami 2017). Various field studies on nectar yeasts give information about yeast cell densities and species composition (Lachance et al.2001; de Vega, Herrera and Johnson 2009; Pozo, Herrera and Bazaga 2011; Álvarez-Pérez and Herrera 2013; Glushakova, Kachalkin and Chernov 2014; Bartlewicz et al.2016). Herrera et al. (2009) investigated 22 different herbaceous plants in Spain and Mexico and concluded that cell densities under natural conditions are likely to be below 4×10^5 cells μl^{-1} in floral nectar. The highest observed cell densities were found in Moraea graminicola with an average of 8.81×10^5 cells μl^{-1} (de Vega, Herrera and Johnson 2009). However, apart from studies about competition and priority effects (Peay, Belisle and Fukami 2012; Vannette and Fukami 2014, 2017; Mittelbach et al.2016), we know little about how cell densities develop over time in flowers that are regularly pollinated.

We here sought to understand how nectar yeasts can develop such high densities within just a few days if they become decimated during every pollination event. To do so, we used a combined laboratory and simulation modeling study. In the laboratory experiments, we determined the 'remaining fraction' of nectar yeasts after nectar removal by an artificial pollinator, and used honeybees to determine the number of 'transported' and finally 'transmitted yeast cells' from one flower to the next. The results of these experiments directly fed into a simulation model, in which the population dynamics and dispersal potential of nectar yeasts were systematically assessed under various scenarios.

The model results may contribute to understanding adaptions to and population dynamics of systems where local populations need to persist during disturbance or dispersal events as well as compensate for periods of high mortality or emigration. These conditions can be found in a variety of systems, including stream drift, floods or wind drift or in microbial communities of water tanks of Bromeliads, in puddles or ponds.

1.3 Materials and Methods

Before planning the lab experiments, we conducted a sensitivity analysis (see supplementary material A in the appendix) of the model described below to identify the

variables with greatest impact on yeast population size: 'growth rate', 'pollination chance' and 'remaining fraction', i.e. the percentage of yeast cells remaining after a pollination event. Data for 'growth rate' and 'pollination chance' could be taken from the literature. The 'remaining fraction' was investigated in Experiment A. In Experiment B, we determined the number of 'yeast cells transported' by the pollinator to better understand the dispersal process. Experiment C was conducted to determine two additional model parameters that have never been reported before: 'dispersal probability' and 'number of dispersed cells'.

For all experiments, we chose two ascomycete yeasts that specialize on sugar-rich environments: *M. reukaufii* (M.) and *C. rancensis* (C.) (Brysch-Herzberg 2004; Belisle, Peay and Fukami 2012; Peay, Belisle and Fukami 2012; Mittelbach et al. 2015, 2016). The strains used here are deposited at Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures under accession numbers 'DSM100740' (M.) and 'DSM100742' (C.).

Experiment A: remaining fraction

In Experiment A, we measured the 'remaining fraction', which is the proportion of yeast cells that remain in a flower when nectar is removed. To mimic this process experimentally, we used the wells of two 96-well plates (greiner bio one, cat# 655180, Frickenhausen, Germany) to represent flower cups. On each plate, we cultivated the two yeast species with four different nectar exchange treatments plus control without yeasts. Each treatment was replicated in eight wells on each plate. Yeasts were grown in artificial nectar (25% sucrose w/v solution supplemented with 0.32 mM amino acids from casein hydroxilate) starting with a density of 20 cells μl^{-1}. Nectar was exchanged never, once (after 48 h), twice (additionally after 72 h) or three times (additionally after 96 h), representing pollination events. Even if nectar exchange times can be much faster in real flowers, we used 24 h in between to ensure recovery of high cell densities before the next nectar exchange. For nectar exchange, the entire artificial nectar was removed with pipettes and fresh artificial nectar was added. To test for the effects of agitation (mimicking the physical effect of a pollinator), one of the two 96-well plates was shaken for 10 s before nectar removal. Before and after each nectar exchange, optical density was measured at 660 nm with a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories GmbH, Munich, Germany).

Experiment B: transported cells

In Experiment B, we measured how many yeast cells stick to the proboscis of a bee when drinking nectar. Cells on the proboscis are 'transported cells' that can be potentially dispersed. We measured the number of 'transported cells' after drinking 10 µl nectar containing a defined density of cells of one of the two yeast species (10^2, 10^3 and 10^4 cells μl^{-1}).

We replicated every treatment with five bees. After the bee emptied the cup, it was killed with CO_2 gas. Probosci of the bees were removed with forceps and vortexed with 50 µl sterile water to separate yeast cells from the proboscis. Every proboscis together with the water was spread on 1% sugar agar plates (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% dextrose, 2.0% agar, 95.9% water, values in % w/w). Agar plates with samples were incubated at 25°C in the dark. After 5 days, the number of colony-forming units (CFUs) was counted.

Experiment C: dispersal probability and proportion of dispersed cells

Experiment C was designed to measure how many yeast cells are dispersed by a pollinator from one source flower to the first and the second consecutive visited flower (Fig. 3). We used honeybees and a plexiglass tunnel with five consecutive chambers that could be handled from outside the tunnel. Bees were placed into the tunnel and passed the chambers. In chambers 2 to 5, we placed the cap of a PCR Eppendorf tube with 10 µl of 25% w/v sucrose solution. In the third chamber, we added one of the two yeast species with the same cell densities as used in Experiment B. The caps in chambers 4 and 5 will be called cup 1 and cup 2 and represent artificial flowers. For every yeast species and cell density, 15 different bees were used. After the bee drunk the medium, the caps were flushed with 50 µl sterile water, streaked on agar plates and incubated, and CFUs of yeasts were counted. In chamber 2, we measured how many yeast cells the bee already carried before the experiment, and in chambers 4 and 5 how many yeast cells the bee dispersed after experimental yeast uptake from chamber 3 to the first (cup1) and second (cup2) visited cup. For detailed method description of lab experiments, see supplementary material B in the appendix.

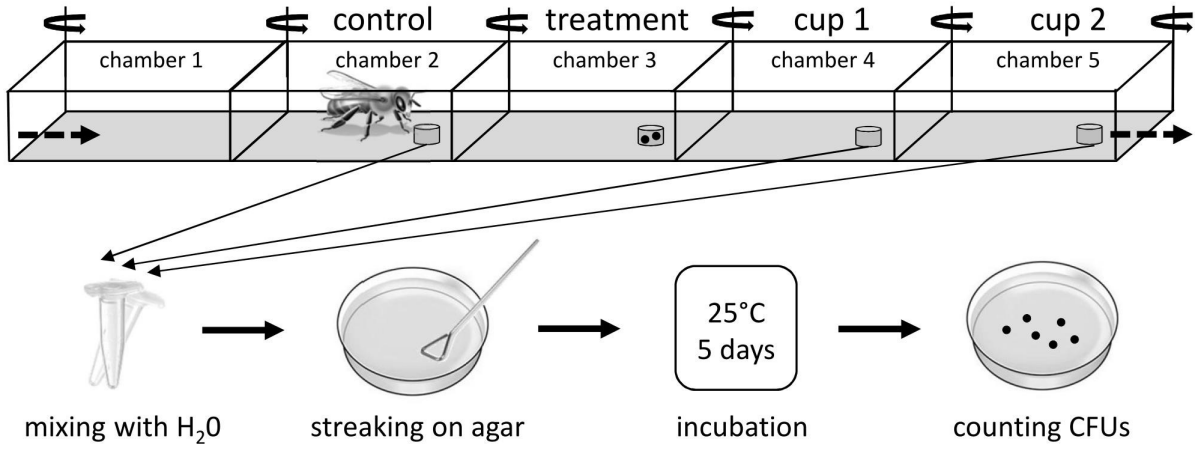


Figure 3: Design of experiment C to measure proportion of dispersed nectar yeast cells. Honeybees passed the tunnel and drank 10 µl artifical nectar each in the chambers 2 to 5. In chamber 3, yeasts were added to the nectar. Remaining cells from the cups were mixed with water, streaked on agar and incubated, and CFUs were counted. Two nectar yeast species in three different densities with each 15 replications were tested.

Simulation model and its parameters

To understand the effect of many consecutive pollination events on population size and dispersal potential, we developed a stochastic simulation model (NetLogo 5.3.1; Wilensky 1999) of nectar yeasts in one single flower. The model calculates the population size and the amount of dispersed cells of a single nectar yeast population over time, dependent on pollination time and chance, inoculated cells during first pollination event, transmitted cells to the next flower, cells that remain in the flower during pollination, nectar production rate and growth rate of yeast cells with lag phase.

To simplify the model and to focus on our main question, we did not add additional factors or traits that can affect yeast growth like temperature or nectar sugar and nitrogen concentration. The effect of such additional factors can be indirectly evaluated by changing the model parameter 'growth rate'.

Most parameter values could be taken from the literature (for details, see supplementary material A2 in the appendix). 'Remaining fraction', 'dispersal probability' and 'number of dispersed cells' were taken from Experiments A and C.

Model experiments

From the sensitivity analysis of the model, we knew that 'growth rate', 'pollination chance' and 'remaining fraction' have the greatest impact on yeast population size. We therefore systematically analyzed the effects of these three variables plus an additional

variable for inoculated cell numbers on yeast performance in our simulations. Inoculated cell numbers were calculated based on results from Experiment C and realistic 'source populations' of 10^3 (low), 10^4 (intermediate) and 10^5 (high) cells μl^{-1} resulting in absolute inoculation numbers of 2, 18 and 175 cells μl^{-1} if inoculation is successful. The range of the simulated 'growth rate' is taken from experiments with artificial nectar with 25% to 40% sugar concentrations as well as six different nectar yeast species (Experiment A, Mittelbach et al2015, 2016) and is reported as % growth per hour. 'Pollination chance' is expressed in the model as % chance per hour. 'Remaining fraction' is given in % of the current yeast population.

We performed model simulations to determine the impact of three uncertain but sensitive parameters ('growth rate', 'pollinator frequency' and 'remaining fraction') on the performance of the yeast population. Each of the three sensitive parameters was separately varied, while keeping the other two sensitive parameters fixed at their default value (see below). This was done for three levels of inoculated cells.

Changes in 'growth rate' were tested from 5% to 23% in steps of 1%; for 'pollination chance' from 0% to 20% in steps of 2%, from 20% to 40% in steps of 5% and from 40 to 100% in steps of 10%; for proportions of 'remaining fraction' from 0% to 100% in steps of 10. We did 1000 repetitions per setting. Default values were 10% 'growth' per hour, 33% 'pollination chance' per hour and 30% 'remaining fraction'. One additional analysis was run testing change in 'growth rate' with optimized fixed value for 'remaining fraction' of 50% which is close to the highest value found in Experiment A for C. (not shaken). Highest population size and total number of dispersed cells (to the next first visited flower) were measured per repetition. For model description, input data, default values and sensitivity analysis, see supplementary material A in the appendix.

Data analysis

We statistically analyzed the data using R, version 3.3.2 (R Foundation for Statistical Computing, 2016).

Experiment A: The 'remaining fraction' was calculated as the ratio between optical density (OD) after and before nectar exchange. Control values were calculated from treatments without nectar exchange. Four outliers had to be removed from the dataset because the 'remaining fraction' was much higher than 1, which was caused by

extremely low OD values below the accuracy of OD measurement. Residuals of the 'remaining fraction' were normally distributed. A linear model was used with 'remaining fraction' as dependent variable and yeast species (M., C.), nectar removal treatment (one, two and three times) and shaking (yes or no) as independent variables.

Experiment B: The 'proportion of transported cells' was calculated as the ratio of counted CFUs from proboscis to the total number of cells taken up. Residuals of 'proportion of transported cells' were normally distributed. To test for significant differences, a linear model was used with 'proportion of transported cells' as dependent variable and yeast species (M. and C.) and density of yeasts cells (10^2, 10^3 and 10^4 cells μl^{-1}) as independent variables.

Experiment C: The 'dispersal probability' was calculated from presence-absence data for yeast cells transported. Residuals of 'dispersal probability' had a binomial distribution. The significance level for 'dispersal probability' was tested with a binomial general linear mixed model (GLMM) with presence-absence data as dependent variable and species (M. or C.), cup (cup1 or cup2) and cell density (10^2, 10^3 and 10^4 cells μl^{-1}) as independent variables.

The 'proportion of dispersed cells' was calculated as the ratio of counted CFUs to the total number of cells taken up. Residuals of 'proportion of dispersed cells' followed a binomial distribution. To test for significance, we used a binomial GLMM with 'proportion of dispersed cells' as dependent variable and the same independent variables as above.

Simulation model: Both data of highest cell density and total dispersed cells showed zero inflation because we tested extremely low and high values of 'growth rate', 'remaining fraction' or 'pollination chance' resulting in low population numbers. The residuals for highest cell density and total dispersed cells followed a quasi-Poisson distribution due to zero inflation. The statistical analysis of the modeled data was done using a GLMM with quasi-Poisson distribution. Highest cell density and total dispersed cells showed a unimodal response, and thus their squared values were used as dependent variables with growth rate, remaining fraction, pollination chance and inoculated cell number as independent variables.

1.4 Results

Experiment A: remaining fraction

The 'remaining fraction' was measured as the proportion of cells remaining in a cup when nectar was removed. For both species, shaking decreased the remaining fraction significantly by 5.1% for M. and by 18.1% for C. ($P < 0.001$, Table 1). The results show mean values for 'remaining fraction' from 46.2% (±3.9% SE) for M. and 51.2% (±11.9%) for C. when samples were not shaken. When shaken, 41.2% (±4.6%) (M.) and 33.1% (±14.5%) (C.) % of cells remained in the wells, with species differing significantly ($P < 0.001$).

Table 1: Results for linear models of remaining fraction (Experiment A), proportion of transported cells (Experiment B) and for the binomial models of dispersal probability and proportion of dispersed cells (Experiment C) and their explanatory variables. 'Species' differentiates between *Metschnikowia reukaufii* and *Candida rancesis*. In Experiment A, 'removal' means the number of consecutive nectar exchange from 1 to 3 and shaking differs between treatments that were shaken and not shaken before nectar exchange. In Experiments B and C, 'densities' means cell density with three levels (10^2, 10^3, 10^4 cells μl^{-1}). 'Cup' in Experiment C distinguishes between the cup that was visited first and second by the honeybee. Num d.f.: numerator degrees of freedom; Den d.f: denominator degrees of freedom. F value is given only for linear models of Experiments A and B. Deviance is given only for binomial models of Experiment C.

Dependent variable	Independent variables	Num d.f.	Den d.f.	F value / Dev	p value
Experiment A					
Remaining fraction	species	1	80	42.9	<0.001
	removal	2	80	15.1	<0.001
	shaking	1	80	7.9	<0.001
	species : removal	2	80	0.4	0.671
	species : shaking	1	80	1.3	0.264
	removal : shaking	2	80	7.4	0.001
	species : removal : shaking	2	80	3.6	0.031
Experiment B					
Proportion of transported cells	species	1	26	0.5	0.489
	density	1	26	1.7	0.198
	species : density	1	26	28.6	<0.001
Experiment C					
Dispersal probability	species	1	178	249	0.655
	density	1	177	249	0.696
	cup	1	176	241	0.004
	species : density	1	175	240	0.255
	species : cup	1	174	235	0.022
	density : cup	1	173	234	0.881
	species : density : cup	1	172	233	0.216
Proportion of dispersed cells	species	1	89	10948	<0.001
	density	1	88	10784	<0.001
	cup	1	87	10777	0.012
	species : density	1	86	10713	<0.001
	species : cup	1	85	10137	<0.001
	density : cup	1	84	10137	0.438
	species : density : cup	1	83	9971	<0.001

Experiment B: transported cells

We calculated the 'proportion of transported cells' as the ratio of number of cells from the proboscis to number of cells taken up before by the honeybee. For C., the 'proportion of transported cells' increased with yeast cell uptake. For M., there was no such pattern. The 'proportion of dispersed cells' differed significantly between yeast species ($P < 0.001$, Table 1). Bees that took up C. in a density of 10^2 cells μl^{-1} transported 0.26% (±0.06% SE) of the cells taken up, for 10^3 cells μl^{-1} it was 3.45% (±0.77%) and for 10^4 cells μl^{-1} it was 9.89% (±1.94%). Bees that took up M. in a density of 10^2 cells μl^{-1} transported 5.28% (±1.65%) cells, 8.93% (±1.80%) cells at 10^3 cells μl^{-1} density and 1.85% (±0.25%) cells at 10^5 cells μl^{-1} density. The average 'percentage of transported cells' over all three cell densities was 5.35% (±1.08%) for M. and 4.53% (±1.25%) for C. ($P = 0.489$). Absolute numbers of transported cells varied between 2 and 14 666 for C. and 12 and 2770 for M. depending on fed cell density.

Experiment C: dispersal probability and proportion of dispersed cells

To describe yeast dispersal from a source flower to the first and second consecutive visited flower, we measured two values: 'dispersal probability' and 'proportion of dispersed cells'. 'Dispersal probability' is the proportion of trials with yeasts present in the first or second visited flower after cell uptake. The 'proportion of dispersed cells' is the ratio between dispersed cells and cells taken up before, and was only measured if dispersal was successful.

In half (49.4%) of the cup visits, the bees did not disperse any yeast cells, independent of the fed cell density. Cell densities had no effect on 'dispersal probability' ($P = 0.696$, Table 1). There was a significant decrease in average 'dispersal probability' over all densities from the first to the second cup. 'Dispersal probability' for M. was 71.1% (±6.8% SE) for the first and 33.3% (±7.1%) for the second cup, for C. It was 51.1% (±7.5%) for the first and 46.7% (±7.5%) for the second ($P = 0.004$). Taking both cups together, average 'dispersal probability' for M. was 52.2% (±5.3%) and for C. 48.9% (±5.3%) ($P = 0.655$).

The 'proportion of dispersed cells' did not change significantly between the three cell densities ($P = 0.696$, Table 1); nevertheless, values were significantly higher for the first than for the second cup across both species ($P < 0.012$). The average 'percentage of

dispersed cells' over all cell densities for M. was 0.282% (±0.060% SE) at the first and 0.177% (±0.059%) at the second cup. If bees took up C., the 'percentage of dispersed cells' was 0.245% (±0.048%) at the first and 0.232% (±0.050%) at the second cup. Species showed significantly different results ($P < 0.001$). Considering both cups together, there was a slightly higher 'proportion of dispersed cells' for M. with 0.248% (±0.045%) than for C. with 0.239% (±0.034%). Absolute numbers of dispersed cells varied between 1 and 444 for C. and 1 and 1105 for M. depending on fed cell density. For detailed results of laboratory experiments, see supplementary material C in the appendix.

To summarize our findings, Fig. 4 shows the absolute cell numbers and proportion of cells at different dispersal steps if the total source population is 104 cells for the two yeast species M. or C. Proportion of cells taken up during nectar foraging is higher for C., but the percentage of cells that stick to the proboscis is higher for M. The 'percentage of dispersed cells' from cells taken up and 'dispersal probability' is higher for M. at the first flower, but higher for C. at the second flower. In the end, absolute dispersed cells for the first and second flower are higher for C. with 19 and 18 than for M. with 17 and 10 cells.

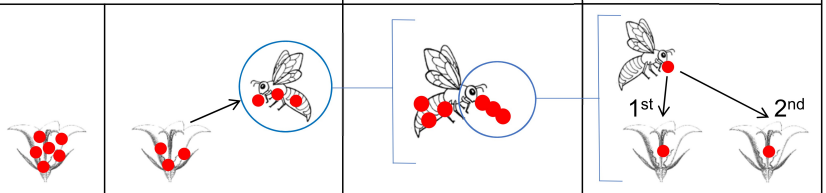
	cells in flower (experiment A)			**transported cells** (experiment B)		**cells dispersed** (experiment C)	
							
Metschnikowia reukaufii	total	remaining	uptaken	swallowed	on proboscis	1st flower 71.1% chance	2nd flower 33.3% chance
absolute cell number	10 000	4120	5880	5565	315	17	10
fraction of cells in flower (%)	100.00	41.20	58.80	55.65	3.15	0.17	0.10
fraction of uptaken cells (%)	-	-	100.00	94.65	5.35	0.28	0.18
fraction of transported cells (%)	-	-	-	-	100.00	5.40	3.17
Candida rancensis						51.1% chance	46.7% chance
absolute cell number	10 000	3310	7690	7342	348	19	18
fraction of cells in flower (%)	100.00	33.10	76.90	73.42	3.48	0.19	0.18
fraction of uptaken cells (%)	-	-	100.00	95.47	4.53	0.25	0.23
fraction of transported cells (%)	-	-	-	-	100.00	5.45	5.17

Figure 4: Detailed proportions and absolute cell numbers of different steps of the dispersal process of nectar yeasts with data from lab experiments. Values are given as averages for *Metschnikowia reukaufii* and *Candida rancensis*. Chance means dispersal probability.

Simulation of yeast population size and dispersal rate

To understand under which conditions a population can reach cell densities of ecological relevance (5 × 10^4 cells μl^{-1} and higher: Herrera, Garcia and Pérez 2008; Herrera and Pozo 2010; Herrera, Pozo and Medrano 2013; Schaeffer and Irwin 2014; Schaeffer et al.2014), we used a stochastic simulation model. The default values of the variables do not represent a specific yeast species but average trait levels over many species.

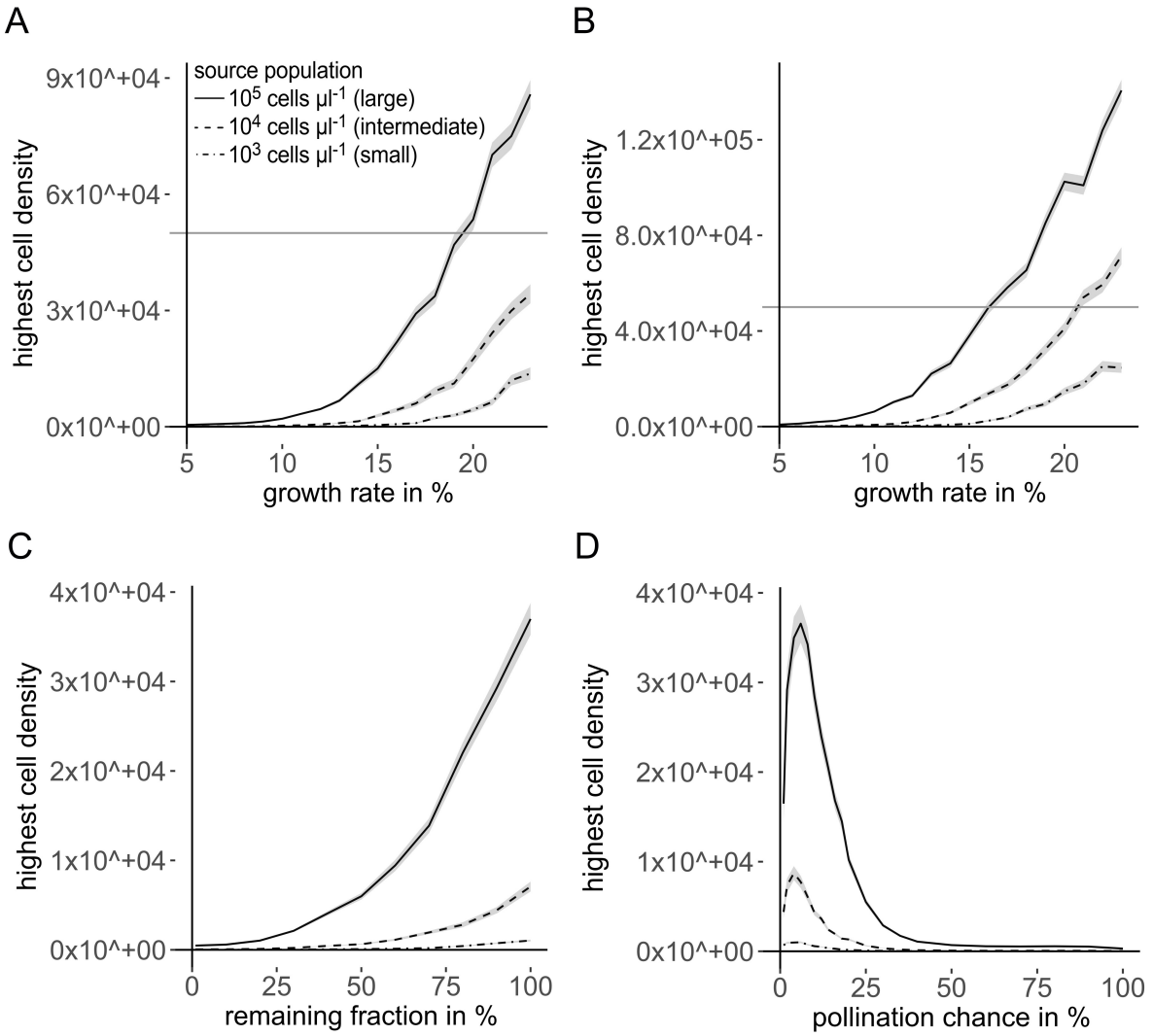


Figure 5: Results of highest cell density for simulations of different steps of growth rate (A and B), remaining fraction (C) and pollination chance (D). For A, C and D: default values for parameters that were not altered on the x-axes are 10% growth rate, 30% remaining fraction and 33% pollination chance. For B: default value of remaining fraction is increased to 50%. The horizontal line in A and B represents target cell density of 5 × 10^4 cells μl^{-1}. The dotted line shows simulation with low source population (10^3 cells μl^{-1}), the dashed line with middle source population (10^4 cells μl^{-1}) and the normal line with high source population (10^5 cells μl^{-1}). The error shadow represents standard error.

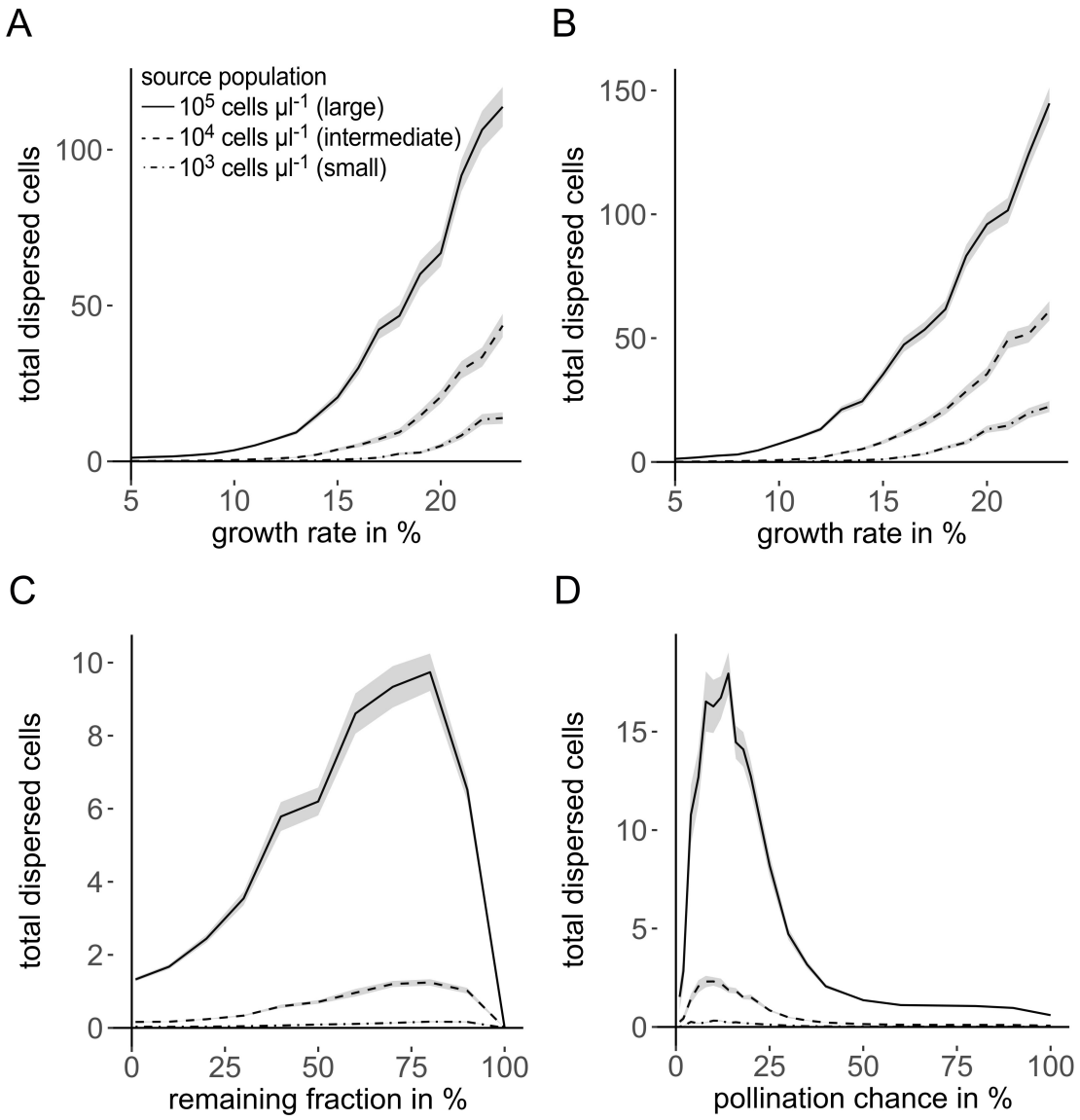


Figure 6: Results of total dispersed cells for simulations of different steps of growth rate (A and B), remaining fraction (C) and pollination chance (D). For A, C and D: default values for parameters that were not altered on the x-axes are 10% growth rate, 30% remaining fraction and 33% pollination chance. For B: default value of remaining fraction is increased to 50%. The dotted line shows simulation with low source population (10^3 cells μl^{-1}), the dashed line with middle source population (10^4 cells μl^{-1}) and the normal line with high source population (10^5 cells μl^{-1}). The error shadow represents standard error.

Population size does not reach target cell density for any 'pollination chance' level with default values (10% 'growth rate', 30% 'remaining fraction', Fig. 5D). Highest cell densities developed with a large source population to 3.6×10^4 cells μl^{-1}, with intermediate source population to 9×10^3 cells μl^{-1} and with small population size to 10^3 cells μl^{-1}.

The higher the 'initial cell number (source population)', the higher the resulting cell density (Fig. 5; $P < 0.001$, Table 2) and the total amount of dispersed cells (Fig. 6; $P < 0.001$, Table 2). A 10 times higher 'source population' increased the mean population size 2 to 10 times. The higher the effect was, the lower the 'growth rate' or 'remaining fraction' was. Both 'growth rate' (Fig. 5A and B; $P < 0.001$, Table 2) and 'remaining fraction' (Fig. 5C; $P < 0.001$, Table 2) increased cell density exponentially. 'Pollination chance' showed an optimal range for highest cell density with a peak at 4% 'pollination chance' for intermediate source population and 6% 'pollination chance' for small and large source populations (Fig. 5D; $P < 0.001$, Table 2). Dispersed cells also increased exponentially with higher 'growth rate' (Fig. 6A and B; $P < 0.001$, Table 2). 'Remaining fraction' showed an optimal range for dispersed cells at around 80% independent of the level of 'initial cells' (Fig. 6C; $P < 0.001$, Table 2). 'Pollination chance' showed the highest effect on dispersed cells at 14% 'pollination chance' for large source populations and 10% 'pollination chance' for intermediate and small population sizes (Fig. 6D; $P = 0.024$, Table 2).

The target cell density of 5×10^4 cells μl^{-1} can be reached with large 'source population' and a 'growth rate' higher than 20% but not with a small or intermediate 'source population' (Fig. 5A). If we set 'remaining fraction' to 50% (instead of our default value of 30%), target cell density can be reached with a 'growth rate' of only 16.5% and large source population or a 'growth rate' of 21% and intermediate source population (Fig. 5B).

The target cell density cannot be reached at any level of 'remaining fraction' with default values (10% 'growth rate', 33% 'pollination chance', Fig. 5C). The highest cell density reached is 3.7×10^4 cells μl^{-1} (large source population), 7×10^3 cells μl^{-1} (intermediate source population) and 10^3 cells μl^{-1} (small source population).

Table 2: Results of general linear mixed models and quasi-Poisson distribution of modeled data for squared highest cell density and squared total dispersed cells against growth rate (5%–25%), remaining fraction (1%–100%), pollination chance (1%–100%) and source population (10^3, 10^4, 10^5 cells μl^{-1}). Num d.f.: numerator degrees of freedom; Den d.f: denominator degrees of freedom.

Dependent variable	Independent variables	Num d.f.	Den d.f.	Dev.	p value
highest cell density	growth rate	1	221998	2.8231e+15	<0.001
	remaining fraction	1	221997	2.7516e+15	<0.001
	pollination chance	1	221996	2.6620e+15	<0.001
	source population	1	221995	2.2968e+15	<0.001
	growth rate : remaining fraction	1	221994	2.2967e+15	0.522
	growth rate : source population	1	221993	2.2683e+15	<0.001
	remaining fraction : source population	1	221992	2.2666e+15	<0.001
	pollination chance : source population	1	221991	2.2660e+15	<0.001
	growth rate : remaining fraction : source population	1	221990	2.2654e+15	<0.001
total dispersed cells	growth rate	1	221998	5095954884	<0.001
	remaining fraction	1	221997	5094350498	<0.001
	pollination chance	1	221996	5085424596	<0.001
	source population	1	221995	4383567883	<0.001
	growth rate : remaining fraction	1	221994	4382035372	<0.001
	growth rate : source population	1	221993	4359520760	<0.001
	remaining fraction : source population	1	221992	4354281131	<0.001
	pollination chance : source population	1	221991	4353924127	0.024
	growth rate : remaining fraction : source population	1	221990	4353924125	0.995

1.5 Discussion

Our experiments and simulations were approaches to understand nectar yeast dispersal rates and temporal population dynamics. We described an ephemeral, complex system with a simple model and generated data for key variables in simple laboratory experiments. The answer to the question how nectar yeasts can develop cell densities with ecological effects higher than 5×10^4 cells μl^{-1} has two parts. First, the 'pollination chance' must be in a beneficial range: high enough to ensure yeast cell inoculation, but low enough to keep decimation events small. Second, yeasts must have a beneficial trait combination of 'growth rate' and 'remaining fraction'. Reaching the target cell density from an intermediate source population is not possible if one trait is too small even if the other one is at maximum. If both traits are present at least at an intermediate level, the system can reach the target cell density. In general, a higher 'growth rate' is always

better, but 'remaining fraction' should not exceed 80% (Fig. 6C) in the described system to allow both a high population growth and high number of dispersed cells.

For the first time, we could quantify nectar yeast cell numbers at different steps of this movement ecology system, and model local nectar yeast population dynamics. Our results can be used to make predictions about nectar yeast growth and consequently changes in nectar chemistry, flower scent and even pollinator behavior.

Pollination chance

'Pollination frequency' can be very different between plant species and ecosystems and has a huge impact on population size and dispersed cells of nectar yeasts. Population growth of nectar yeasts is logistic without repeating pollination events as shown in the conceptual Fig. 7. Even if the pollinator is needed for inoculation and dispersal, it can be a detriment to population growth if pollination occurs too often: high pollination frequency reduces populations faster than growth can compensate and consequently population size stagnates or even decreases. A 'pollination frequency' that is relatively low permits large population growth and consequently a high potential number of dispersed cells, but this also strongly reduces 'dispersal probability'. In our simulations, pollination was restricted to 10 h per day. Therefore, our results are only applicable to flowers that are pollinated only at daylight or only at nighttime. The long times without pollination are necessary for nectar yeasts to recover high cell densities.

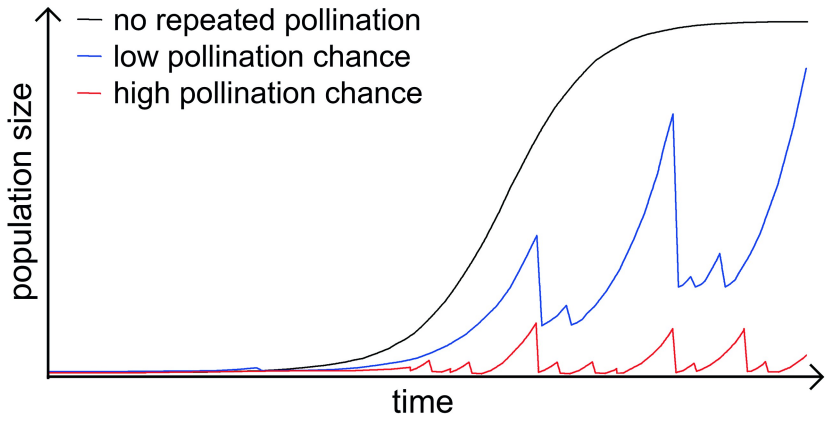


Figure 7: Schematic development of population size of a local nectar yeast colony over time with just one pollination event in the beginning (black line), low supporting (blue line) and too high hindering (red line) pollination chance. Population decimations are caused by nectar extraction during pollination events.

Highest positive impact on population size and dispersed cells was found in the model between 5% and 20% 'pollination chance' per hour, which equals a pollination event every 5 to 20 h. If we consider no pollination at 14 h per day in our model, that means only one pollination event every 1 or 2 days. This is similar to the rates observed in temperate rain forests of Chile by Martinez and Armesto (2005), but not representative of European ecosystems where pollination occurs every 1.5 h (Nedić et al.2013) to 4.2 h (Hausmann, Petermann and Rolff 2016). Default value for 'pollination chance' in our simulations was set to 3.0 h (33% 'pollination chance') to simulate an intermediate and realistic pollinator density for Europe.

Our simulation model ran with a maximum 'flower lifetime' of 4 days which is representative of many flowers even if some species can remain open much shorter or longer timespans (supplementary material A2, appendix Table 4). In our sensitivity analysis (supplementary material A3 in the appendix), we tested the effects of different 'flower lifetimes' from 1 to 10 days and found only very small effects on 'highest cell density' compared to other sensitive parameters. We did not take into account the effect of the pollination duration a pollinator spent on a single flower or cup in Experiment C to keep the model as simple as possible. A longer and therefore more precise foraging time may decrease the remaining fraction, and nectar yeasts would need an even higher growth rate to compensate for that.

Besides dispersal, pollination can have other positive effects on local yeast populations. Foraging of nectar removes also accumulated secondary metabolism products of the yeasts such as aliphatic alcohols, glycosides (aucubin, catalpol, ouabain), alkaloids (nicotine) or methylxanthines (caffeine) (Golonka, Johnson and Hinson 2014; Vannette and Fukami 2016), which may have negative effects on growth of nectar yeasts. In addition, with uptake of nectar the pollinator triggers the flower to increase production of fresh nectar resources (Nicolson, Nepi and Pacini 2007). Mittelbach, Yurkov and Begerow (2016) discussed the effects of different changes in pollinator behavior to nectar yeasts and concluded that a change in foraging behavior of insects decreases nectar yeast diversity and dispersal in metapopulations. Our results also showed that too high a pollination chance is detrimental to a local yeast population but if pollinator density is too low a preference of pollinators for yeast-infected flowers can also have

positive effects on local nectar yeast population size as long as pollination chance remains in the beneficial range.

Remaining fraction

With high 'pollination chance', the decimation of yeasts must be reduced to allow yeast population survival. Important for the development of nectar yeast populations is the amount of cells that resists the extraction with nectar by pollinators and remains in the flower. For the nectar yeast population size, it is beneficial if more cells remain in the flower, but then fewer cells are also available for dispersal. We found that a value of 80% 'remaining fraction' allows the highest possible number of cells available for population growth without reducing the total number of dispersed cells during the whole flower lifetime (Fig. 6A). We assume that it is realistic to have 80% 'remaining fraction' in a natural flower with complex structures and hairs because we already found up to 51% 'remaining fraction' in a flat well in Experiment A. We observed that yeast cells in Experiment A sank to the bottom of the 96-well plate and formed a layer similar to a biofilm but not that resistant. The layer could not be completely dissolved with the shaking intensity used, but more intense shaking could dissolve the layer completely after the experiment. We hypothesize that if yeast cells maximize growth as predicted by the model, we expect that they may exhibit adaptation for adhesion, thus resisting removal. The 96-well plate had a hydrophobic surface. Flower surfaces can have very different hydrophobicity (Feng et al.2011) that may influence attachment. Biofilm formation in a clinical context has been reported for *Candida auris* and *C. haemulonii* that are closely related to M. and C. in the Metschnikowiaceae clade (Oh et al.2011).

The target cell density of the model (5×10^4 cells^{-1}) is based on studies working on plants that are mainly bumblebee pollinated. Nevertheless, we conducted our laboratory Experiments B and C with the commonly used pollinator model organism honeybee. For bigger pollinators like bumblebees or even birds, we would expect the remaining fraction to increase due to small flower structures not accessible with bigger mouthparts. Additionally, we assume the number of dispersed cells to increase because transported nectar amounts are likely higher. The sensitivity analysis (supplementary material A3 in the appendix) showed that the number of dispersed cells has only a very small effect on the results because logistic growth of the yeasts overrides the effect of a comparably small number of added cells. On the other hand, the remaining fraction has

large effects and we tested it as a sensitive parameter in our main results (Figs 5C and 5C). It remains uncertain by how much the remaining fraction will increase if main pollinators have bigger or different mouthparts than honeybees.

Transported cells and inoculation

Even if large amounts of yeast cells are extracted with the nectar, around 95% of yeast cells will be swallowed and only 5% stick to the bee's proboscis (Fig. 4). In Experiment B, M. showed a slightly higher trend for transported cells than C., which may be explained by the Y-cell conglomerates that may increase the sticking to the hairs of the honeybee proboscis (Brysch-Herzberg 2004). Inoculation of yeast cells is not guaranteed if a bee that transports yeast cells on its proboscis visits a flower. We observed no correlation between the number of cells a bee was fed and the chance that inoculation to the next cup was successful in Experiment C. We believe this is due to the honeybee proboscis that consists of labellum, glossa, paraglossa and labial palpus each with different hair sizes and densities. Yeast cells floating in consumed nectar pass these structures, accumulate and form conglomerates in some spots. When pollinating the next flower, yeast cells are unlikely to be released as single cells rather than whole conglomerates. The size of the conglomerate still depends on the number of cells taken up. Our results indicate that dispersal of single nectar yeast cells has only little chance of success, can be measured and is predictable with a few factors such as 'pollination chance', chance that at pollinator transports yeast cells, 'dispersal chance' and 'proportion of dispersed cells'.

Transferability to other ecological systems

Beside yeasts, a large part of the microbial community in nectar consists of bacteria (Fridman et al.2012; Álvarez-Pérez and Herrera 2013). Bacterial growth rates and inoculation densities are likely to be far higher than for nectar yeasts. Consequently, they may compensate for population loss much more readily than nectar yeasts. The threshold for bacterial ecological effects may also differ drastically from that of yeasts.

As we have shown in Experiment A, different nectar yeast species vary in their disturbance tolerance (here: remaining fraction). Villarreal-Barajas and Martorell (2009) showed in plant species of semi-arid grassland along an urbanization gradient that more competitive species showed higher abundance under low disturbance. With

increasing disturbance competitive species decline and disturbance-tolerant species increased, Violle, Pu and Jiang (2010) found a tradeoff between competition ability and disturbance tolerance in a protist model system. Our results suggest that this tradeoff may also exist for nectar yeasts and that disturbance-tolerant species even with low growth rate may be more successful under high pollination pressure than disturbance-vulnerable species with high growth rates. Pollination that includes inoculation, decimation and dispersal for nectar yeasts is more similar to mass emigration events with a high mortality during migration like stream drift (Waters 1972; Brittain and Eikeland 1988), floods (Naiman and Décamps 1997) or wind drift (Pedgley et al.1990). It is also in some ways similar to plant biomass reduction by grazing herbivores that also transport seeds (Collins and Uno 1985; Couvreur et al.2004). Another comparable system are Bromeliads and Neotropical plants that form tanks within their leaf axils, in which rainwater accumulates and communities of microorganisms, algae and detritivores emerge, connected by the dispersal of predatory invertebrates (Benzing 2000; Srivastava and Bell 2009; Starzomski, Suen and Srivastava 2010). Also, here invertebrates are the necessary predator and dispersal agent at the same time, shaping the community, and these can also be detrimental to the community if predation occurs too often (Petermann et al.2015). Populations of microorganisms and invertebrates in puddles or small ponds that are inoculated by drinking animals may also respond like nectar yeast populations if these ephemeral aquatic systems are emptied or fall dry (Kushlan 1976). In all these systems, the local population needs to persist during disturbance or dispersal events, compensating for a periodically high mortality or emigration. Our findings here suggest that populations in such situations should have a trait combination of an intermediate persistence ability and intermediate growth rate instead of maximizing either trait alone.

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2 CHAPTER 2: DISPERSING SPECIES POOLS REVEAL HOW MOBILE LINKS SHAPE METACOMMUNITIES OF NECTAR YEASTS

2.1 Abstract

Dispersal between local communities is a hallmark of metacommunity theory. Evidence for the effects of dispersal rates and pathways on metacommunity dynamics under field conditions is often lacking. However, studying metacommunities of yeasts in floral nectar and yeasts transported by a mobile linker community offers the opportunity to understand how species pools of passively dispersing organisms affect the community assembly. We sampled flowers and pollinators of three common tree species, identified attached yeast species and observed flower visits of different pollinator groups. We found a high overlap in yeast communities between flower and pollinator for social insects and low overlap for solitary insects. Bumblebees and honeybees transported the most nectar-specialist species, wild bees and wasps the most insect-associated yeast species, and sawflies the most transient yeast species. We found strong environmental filtering for insect-associated yeast species. Our results show that the dispersal frequency of the mobile linker community plays an important role in determining the species richness and the overall species abundance of the metacommunity, whereas local processes like environmental filtering effects mainly shape species composition. This approach offers new insights into the role of ecological filters during dispersal and colonization processes and a better understanding of resulting metacommunities.

2.2 Introduction

An important part of metacommunity theory is dispersal between local communities (Leibold et al. 2004). Dispersal rates and pathways are mainly inferred from differences between local communities and habitats. While the significance of different dispersal rates and conditions on metacommunity dynamics are tested with experiments and simulations, direct evidence of dispersal processes from observation under field conditions is often lacking (Jacobson and Peres-Neto 2010). One major challenge is to track many individuals of multiple species during dispersal. Overcoming this obstacle would allow us to better understand dispersal processes and community formation under natural conditions.

The metacommunity of nectar yeasts offers a unique opportunity to study passive dispersal by zoochory. Flowers are regarded as separated local patches which are linked majorly by pollinators (De Vega and Herrera 2012; Pozo, Herrera and Alonso 2014; Schaeffer and Irwin 2014) forming the mobile linker community (Lundberg and Moberg 2003). Within single flowers, pollinators additionally cause disturbance by extracting nectar with a part of the yeast populations (Hausmann, Tietjen and Rillig 2017) and trigger the production of new nectar (Nicolson, Nepi and Pacini 2007). Note that we defined a local community as the collection of yeast species existing on all the flowers of an individual plant, even if yeasts in a single flower could be seen as a local community, because single flowers are species-poor containing mostly only one, rarely more different species (Herrera et al. 2010; Pozo, Herrera and Bazaga 2011; Belisle, Peay and Fukami 2012).

Regional processes including the frequent disturbances by pollinators are predicted to be more important than local processes, as shown by Collins, Glenn and Briggs (2002), Belote, Sanders and Jones (2009) and Myers and Harms (2011), whereas local habitat conditions serve as strong environmental filter (Herrera et al. 2010). However, previous studies investigated only small parts of the pollinator guild (Lachance et al. 2001; Rosa et al. 2003; Brysch-Herzberg 2004; Herrera et al. 2010; Mittelbach et al. 2016). No studies have analyzed the whole floral visitor spectrum to gain a better understanding of the structure, diversity, and dispersal of yeast metacommunity particularly from the regional process perspective. It remains unclear how the communities of nectar-occupying yeast species in flowers are assembled. Although there is a small group of yeasts that is ubiquitous in and almost only isolated from floral nectar (*Metschnikowia reukaufii*, *Metschnikowia gruessii* and *Candida rancensis*), often there are transient or insect-associated species found that are primarily associated with other substrates, such as insects or the phylloplane. A key question is how frequent and in which composition those different yeast groups are transported by different pollinator groups.

In order to capture yeast dispersal by pollinators and thus to understand the extent of ecological filtering in the nectar yeast metacommunity, we carried out a comprehensive sampling. We caught a representative number of individuals of all observed groups of pollinating insects on a specific plant species and identified yeasts transported on their proboscis. A set of the yeast species carried by pollinators, we will call ‘dispersing

species pool' for lack of an already established term, even if the classical definition of a species pool includes all species in the environment (Cornell and Harrison 2014).

In the first step we compared species richness, species abundance and composition between the regionally dispersing species pool (pollinator level) and the locally established metacommunities (flower level). In the second step we analyzed how the frequency and specialization of the mobile linker community (i.e. pollinator guild) influence the metacommunity formation. Thus, we focused on the following questions.

1. Do the yeast species richness and abundance from flowers differ from those taken from the pollinating mobile linker community and among different plant species?
2. Does a higher pollinator visitation frequency increase nectar yeast species richness and abundance in flowers?
3. How similar are yeast communities isolated from flowers and isolated from the pollinating mobile linker community?
4. Do specific pollinator groups transport specific nectar yeast species or functional yeast groups?

These multifaceted investigations of pollinating mobile linker community can offer a deeper insight into metacommunity and movement ecology of nectar yeasts and, more generally, the dynamics of frequently disturbed and regional process dominated metacommunity.

2.3 Materials and Methods

Sampling design

Our study was in the Uckermark region in Brandenburg, Germany, northwest of the city of Prenzlau (North-South: 53°23'30"N to 53°18'40"N, East-West: 13°32'20"E to 13°46'50"E). Our sampling took place in the catchment area of the river Quillow, which has a size of 25,704 ha and is dominated by agricultural fields that cover 65.7 % of the area. Forests cover 12.1 % of the area, grasslands 11.6 %, waterbodies 4.4 %, buildings and roads 2.0 % and other landscape types 4.3 %.

In the beginning of April 2016, we chose five freestanding trees of each of three regionally frequent tree species: *Acer platanoides* (*Acer*), *Robinia pseudoacacia* (*Robinia*) and *Tilia platyphyllos* (*Tilia*). Each species has a different flowering time and our study hence captures a significant part of the pollinator season from April to June. The five chosen trees per species were evenly distributed throughout the whole Quillow Catchment (supplementary material A, appendix Fig. 4). Tree height and crown diameter were estimated to select trees of similar size. All tree species are mainly or exclusively insect-pollinated. The flowers of *Acer* and *Tilia* are open and nectar and pollen are freely accessible to all insects. Typical pollinators are beetles, flies, syrphids, wasps and bees (Mueller 1881). *Robinia* has long tubular flowers with hidden nectar and is mainly pollinated by bees (Mueller 1881).

Sampling was done three times per tree individual. Due to cold and rainy weather conditions four of five tree individuals of *Acer* could only be sampled one time and one individual two times. *Acer* was sampled from April 11th to 19th 2016, *Robinia* from June 6th to 12th 2016 and Tilia from June 16th to 23th 2016. Sampling was done between 10 am and 5 pm on warm and sunny days (12 – 24 °C) with low wind speeds (Beaufort scale 0 to 2). Sampling includes taking weather data (temperature, cloud cover and wind strength), measuring sugar concentration of nectar, taking nectar samples, counting pollinator visitation frequency and catching pollinating insects (sampling procedure is illustrated in appendix Fig. 5).

Observation of pollinator visitation frequency

To evaluate the composition of the pollinator (mobile linker) community, we recorded flower visits on trees. Even if we analyzed all insect flower visitors, pollinators and non-pollinating flower visitors, we call them all pollinators in this study for simplification. We first chose three branches at a height of 2–3 m on the sunny side of the tree. Every branch was observed for 10 mins per sampling day from 2 m distance. We first estimated the number of flowers of the observed focal branch. Then we counted the number of flower visits on this branch for those pollinators landing on an inflorescence within 10 mins. The flower visits of pollinators from the three 10-minuts sessions were summed for each tree and day. Then we calculated the number of visits per 1000 flowers per 30 minutes because of differences in the number of observed flowers. In the field, pollinating insects could be morphologically distinguished to the level of:

honeybees (Hymenoptera, *Apis mellifera*), wild bees (Hymenoptera, Apidae, without the genera *Apis*, *Bombus* and *Psythirus*), bumblebees (Hymenoptera, Apidae, genera *Bombus* and *Psythirus*), wasps (Hymenoptera, Apocrita without Apidae), hoverflies (Diptera, Syrphidae) and other flies (Diptera without Syrphidae), beetles (Coleoptera) and butterflies (Lepidoptera). All other insects were grouped together.

Nectar sampling

Six nectar samples from six different flowers were taken per tree and repetition. The samples were taken with 5 µl pre-calibrated capillary pipettes (Vitrex Medicals, Herlev, Denmark). The samples were pipetted in the field to prepared 200µl PCR-tubes (Eppendorf, Hamburg, Germany) with 50 µl sterile water. Nectar amount per flower differed from 1 to 3 µl. In the evening nectar samples were vortexed and streaked on a 1 % sugar agar plate (0.3 % yeast-extract, 0.3 % malt-extract, 0.5 % peptone, 1.0 % dextrose, 2.0 % g agar, 95.9 % water, values in % w/w). The sugar concentration of nectar was measured in the field with a collective sample from five different flowers using refractometers (eclipse 45-81 and eclipse 45-82, Bellingham and Stanley, Kent, UK).

To test if yeast dispersal is restricted to pollinators, one branch with flower buds from one tree per tree species was covered with a mesh bag to exclude pollinators. Open flowers were removed beforehand. One week later we took nectar samples from 10 flowers of the branch and processed it as described above. No nectar samples from flowers covered with mesh bags contained yeasts.

Insect sampling

After recording pollinator visitation frequencies, 5 to 15 pollinators from all present pollinator groups were caught by netting from the flowers during pollination. Caught insects were placed into single sterile glasses and chilled down in a cooling box to 5 to 10 °C. In the evening insects were killed with CO_2 gas and pinned. Their tongues were removed with sterile forceps under a binocular and mixed in 50 µl sterile water. The tongue with the water was spread on agar plates and handled like nectar samples. Even though yeast dispersal is possible by other insect body parts, too, the method was restricted to the mouthpart, because it does most frequently contact the nectar medium.

Molecular yeast identification

Yeast colonies from incubated agar plate samples were transferred to single agar plates for isolation and grown again for 5 days at 25 °C in the dark. Yeasts were morphologically sorted into different morphotypes. Randomly chosen subsamples representing 25 % of one morphotype were identified genetically. If all subsamples were from the same species all other samples from this morphotype were expected to be the same species. If subsamples showed different species all other samples of the morphotype were identified. Amplification was performed based on the D1/D2 domains of the large subunit nuclear ribosomal RNA following (Lachance et al. 1999). For detailed description see supplementary material B in the appendix. At least one strain per yeast species was deposited at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures under accession numbers DSMZ105599 to 105624. Yeast systematics follows (Liu et al. 2015; Wang et al. 2015).

Isolated yeast species were separated into three ecological groups (see supplementary material A, appendix Table 8) depending on their main habitat or the main source of isolations based on species descriptions by Kurtzman et al. (2011): nectar-specialist, insect-associated and transient species.

Statistical methods

Yeast species richness and abundance

All analysis were done using R, version 3.4.3 for Windows (R Development Team 2017). For each individual tree and tree species we calculated yeast species richness and proportions of samples containing at least one yeast species for nectar and insect samples. We also calculated both variables for nectar samples only for the dispersing yeast species pool that was isolated from specific but dominant insect groups (honeybees, bumblebees, wild bees, wasps and sawflies) to test their effect on only the yeast species they transported. We tested for normal distribution of residuals and homogeneity of variances. To answer the first research question (i.e. do the yeast species richness and abundance from flowers differ from those taken from the pollinators community and among different plant species?), we used data of individual trees (n=15) in a linear model with yeast species richness or proportion of samples containing at least one yeast species as dependent variables and type of samples

(pollinators or nectar) and tree species (three species) as independent variables. The linear models were tested with a post-hoc TukeyHSD.

Effects of pollinator visitation frequency

Data of yeast species richness and proportion of samples containing at least one yeast species per single observations were normally distributed but the single observations from different repetitions (n=36) were nested for tree individual (n=15). The second research question (i.e. does a higher pollinator visitation frequency increase nectar yeast species richness and abundance in flowers?) was tested with data from single observations using linear mixed effect models (lme) with tree individual as a random effect, yeast species richness in nectar or proportion of nectar-samples with yeasts as dependent variables and pollinator visitation frequency of different pollinator groups as independent variables.

Similarity between yeast metacommunities

To answer the third research question (i.e. how similar are yeast communities isolated from floral nectar and isolated from the pollinators?), we used the R package 'bipartite' (Dormann, Gruber and Fruend 2008). We corrected the data for Acer for number of repetitions (data times 2.5), because for Acer just 6 observations were available, while there were 15 for each of the two other tree species. Three different datasets were created for bipartite networks: flower visits of nine insect groups on three tree species (plant-pollinator, n=17 614), number of isolates of 19 yeast species from five insect groups (yeast-pollinator, n=266) and number of isolates of nine yeast species from nectar of three tree species (yeast-plant, n=127). Networks were plotted with the function 'plotweb'. We created ten null-models (random 2-way tables with given marginals using Patefield's algorithm) each for the yeast-pollinator and the yeast-plant network to test whether observed networks are significantly different from random networks based on the same number of species and interactions. Averages of network indices from null-models were tested against observed indices with a t-test. In addition, selected bipartite network indices of yeast species for specialization and nestedness in each network were calculated. Note that any network indices could not be statistically derived for the plant-pollinator network because almost all nodes between them were connected.

To visualize similarity in yeast metacommunity composition from nectar and pollinators of different tree individuals and tree species or different pollinator groups from different tree species, we applied a Principal Coordinate Analysis (PCoA) ordination technique using the 'vegan' package (Oksanen et al. 2018). As distance measure, we used the Bray-Curtis dissimilarity.

Associations of yeasts and pollinators

R package 'indicspecies' (Caceres and Jansen 2016) was used to answer the fourth research question (i.e. do specific pollinator groups transport specific nectar yeast species or functional yeast groups?). For the species indicator analysis we used the 'Indval' function with 999 permutations and allowed group combinations to get the probability that the surveyed yeast species belongs to the target tree species (yeast-plant-network, n=35 observations) or to the target pollinator group (yeast-pollinator-network, n=175 observations).

In addition, we used a Geographical Information System and a machine learning approach to analyze how pollinator visitation frequency as a ratio for their abundance is affected by habitat structures around the trees (supplementary material C in the appendix).

2.4 Results

Yeast species richness and abundance

The nectar of 97 out of 204 (47.5 %) flowers contained yeasts. We identified 9 species from 127 yeast isolates from nectar samples. In total 341 pollinators were caught, of which 188 (55.1 %) transported yeasts. We identified 19 species from 266 yeast isolates from these insects. More information about total yeast diversity of nectar and pollinator samples and nectar sugar concentration is given in Table 3.

We found that average yeast species richness in nectar per individual tree (Fig. 8a) increased with progressing season and was smallest for *Acer* with 0.8 (±0.2), intermediate for *Robinia* with 2.4 (±0.2) and highest for *Tilia* with 5.0 (±0.7) yeast species per tree ($p < 0.001$). Average yeast species richness isolated from pollinators per individual tree was similar for *Acer* and *Robinia* with 3.6 (±0.8) and 3.8 (±0.6) ($p = 0.999$) and double that for *Tilia* with 9.2 (±1.0) yeast species per tree ($p < 0.001$).

Collectively, the results indicate that species richness of yeast species in flowers are significantly lower than that taken from pollinators ($p < 0.001$) and dependent on plant species (or season).

Table 3: Nectar and pollinator samples sorted by tree species or insect group with respective number of samples, samples with yeasts, number of yeast isolates, yeast species richness and Shannon diversity and nectar sugar concentration.

Group of samples	**Number of samples**	**Samples with yeasts**	**Number of yeast isolates**	**Yeast species richness**	**Shannon diversity of yeast species**	**sugar concen-tration**
pollinator samples by tree species						
Acer	60	25 (41.7 %)	27	10	2.04	-
Robinia	114	56 (49.1 %)	75	6	1.24	-
Tilia	167	107 (64.3 %)	164	14	2.15	-
pollinator samples by insect groups						
honeybees	93	61 (65.6 %)	96	9	1.81	-
bumblebees	155	81 (56.8 %)	109	12	2.03	-
wild bees	52	29 (55.8 %)	37	12	2.05	-
wasps	15	8 (53.3 %)	9	7	1.89	-
sawflies	19	9 (47.4 %)	15	9	2.03	-
other insects	7	0 (0.0 %)	0	0	-	-
all pollinator samples	**341**	**188 (55.1 %)**	**266**	**19**	**2.21**	**-**
nectar samples						
Acer	30	6 (20.0 %)	6	2	0.45	15.8 % ±1.4
Robinia	84	37 (44.0 %)	42	4	1.08	20.5 % ±5.5
Tilia	90	54 (60.0 %)	79	8	1.60	27.9 % ±1.8
all nectar samples	**204**	**97 (47.5 %)**	**127**	**9**	**1.71**	**21.4 % ±2.9**

Average proportion of nectar samples containing at least one yeast species per individual tree (Fig. 8b) showed the same pattern as species richness (Fig. 8a), with the smallest proportion of 20.0 % (±6.3) for Acer, a higher percentage of 45.0 % (±8.6) for *Robinia* and the highest proportion for *Tilia* with 60.0 % (±9.8) ($p=0.003$). Average proportion of pollinator samples transporting at least one yeast species found at individual trees was similar for *Acer* and *Robinia* with 43.7 % (±6.3) and 46.6 % (±10.9) ($p=0.999$) and slightly higher for *Tilia* with 64.5 % (±4.2) ($p=0.466$). There was no significant difference of incidence between nectar and pollinator samples across all yeast species.

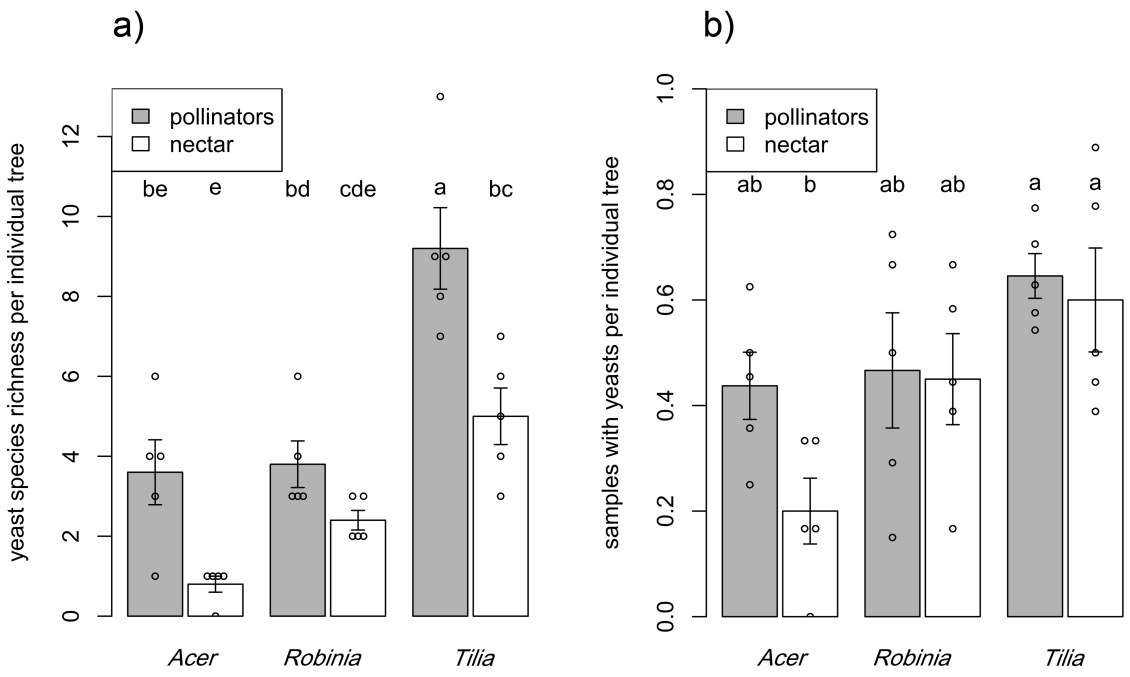

Figure 8: Average yeast species richness (a) and average proportion of samples containing at least one yeast species (b) per individual tree from nectar samples and pollinator samples on three tree species. Error bars represent standard error. Points represent original data. Different letters indicate significant differences after a TukeyHSD based on linear models.

The species accumulation curve of all pollinator samples showed that after finding 19 yeast species from 341 pollinator individuals, a new yeast species would only be expected after sampling 40 additional individuals (supplementary material A, appendix Fig. 7). Species accumulation curves of yeasts isolated from nectar samples suggest that our sampling was sufficient for *Robinia* and *Tilia*, but not for *Acer* because of the limited number of sampling repetitions (supplementary material A, appendix Fig. 6). Species accumulation curves of yeasts isolated from pollinators suggest that our sampling was sufficient for whole pollinator guilds per tree species but not for underrepresented pollinator groups like wild bees, wasps and sawflies (supplementary material A, appendix Fig. 7 and 8) due to their small densities with less than 5% of total flower visits (supplementary material A, appendix Table 9).

Effects of pollinator visitation frequency

The highest pollinator visitation frequency was observed for *Tilia* with 781.3 (±143.1 SE) flower visits at 1000 flowers per 30 mins, followed by *Acer* with 474.7 (±34.3) and 222.8 (±65.5) for *Robinia*. Flower visits were dominated by 50.4 % (±4.7) bumblebees

followed by 21.4 % (±3.1) honeybees and 13.5 % (±3.8) wild bees (supplementary material A, appendix Table 9).

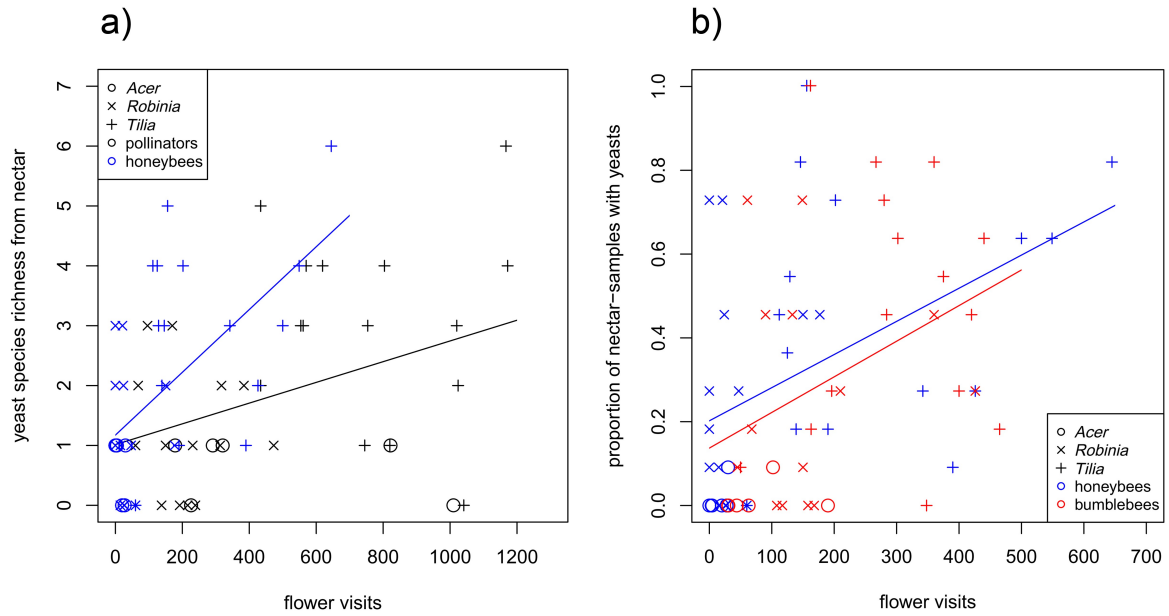

Figure 9: Relationships between flower visits at 1000 flowers over 30 mins against yeast species richness found in nectar (a) and against proportion of nectar-samples containing at least one yeast species (b). Data points represent single observations (n=35) for *Acer* (circles), *Robinia* (crosses) and *Tilia* (plusses). Black data points refer to all pollinator groups, blue data points represent honeybee-data and red data points bumblebee-data. Results from statistical tests are given in Table 4.

Yeast species richness in nectar was positively correlated with overall flower visits (Fig. 9a, p=0.017, Table 4) and only bumblebee-flower visits (p=0.006). The proportion of nectar samples with yeasts was also driven by pollinator visitation frequency. The chance to find yeasts in a flower increased with flower visits of honeybees (Fig. 9b, p=0.008, Table 4) and bumblebees (p=0.024) but not for flower visits of all pollinators together. In addition, we show that both, species richness and abundance of yeasts, are correlated between pollinator and flower level (supplementary material A, appendix Fig. 9 and appendix Table 10).

The surrounding landscape matrix explained most of the pollinator activities as a proxy of the frequency of yeast dispersal, and therefore, the landscape may indirectly explain local yeast abundance and diversity (supplementary material C in the appendix).

Table 4: Results of the linear mixed models for yeast species richness (sp. richn.) and proportions of samples with yeasts (samples w. y.) from nectar using tree ID (n = 15) as a random effect (Fig. 9). Plant distinguishes between the three sampled tree species. Flower visits (FV) are numbers of flower visits per 1000 flowers per 30 mins. Subsets of honeybees (honeyb.) and bumblebees (bumbleb.) represent only the yeast species pool that was transported by the specific pollinator group. num d.f.: numerator degrees of freedom; den d.f: denominator degrees of freedom.

	Dependent variable	Independent variables	Num d.f.	Den d.f.	F value	p value
a)	sp. richn. nectar	FV all	1	14	7.4	0.017
		FV honeyb.	1	14	10.3	0.006
		plant	2	12	3.5	0.064
		FV all * plant	2	14	0.5	0.615
		FV honeyb. * plant	2	14	0.5	0.640
b)	nectar-samples w. y. (honeyb.)	FV honeyb.	1	17	9.0	0.008
		plant	2	12	2.6	0.119
		FV honeyb.* plant	2	17	0.1	0.894
	nectar-samples w. y. (bumbleb.)	FV bumbleb.	1	17	6.1	0.024
		plant	2	12	3.4	0.070
		FV bumbleb. * plant	2	17	0.1	0.999

Similarity between yeast metacommunities

All yeast species found in nectar except for *Sarocladium strictum* were also found on mouthparts of pollinators (Fig. 10c). Eleven yeast species were only isolated from pollinator samples but were not found in floral nectar (Fig. 10b). The yeast species detected from the pollinators belong mainly to M. reukaufii (30.9 %, Fig. 10b), *M. gruessi* (16.3 %), the *M. pulcherrima* clade (9.5 %), and to *Papiliotrema laurentii* (7.8 %). Network interaction indices for pollinator-yeast networks (Fig. 10b) and results of statistical tests against null-models are given in supplementary material A, appendix Tables 11 and 12 (connectance $p<0.001$, weighted nestedness $p<0.001$, interaction evenness $p<0.001$, H´2-value $p<0.001$). The yeast metacommunity from all flowers mainly consist of *M. reukaufii* (39.9 %), *Aureobasidium pullulans* (22.6 %), *P. laurentii* (17.5 %), *M. gruessii* (13.9 %) and *Vishniacozyma victoriae* (10.2 %) (Fig. 10c). Network interaction indices for plant-yeast networks (Fig. 10c) and results of statistical tests against null-models are given in supplementary material A, appendix Tables 13 and 14 (connectance $p<0.001$, weighted nestedness $p=0.181$, interaction evenness $p<0.001$, H´2-value $p<0.001$).

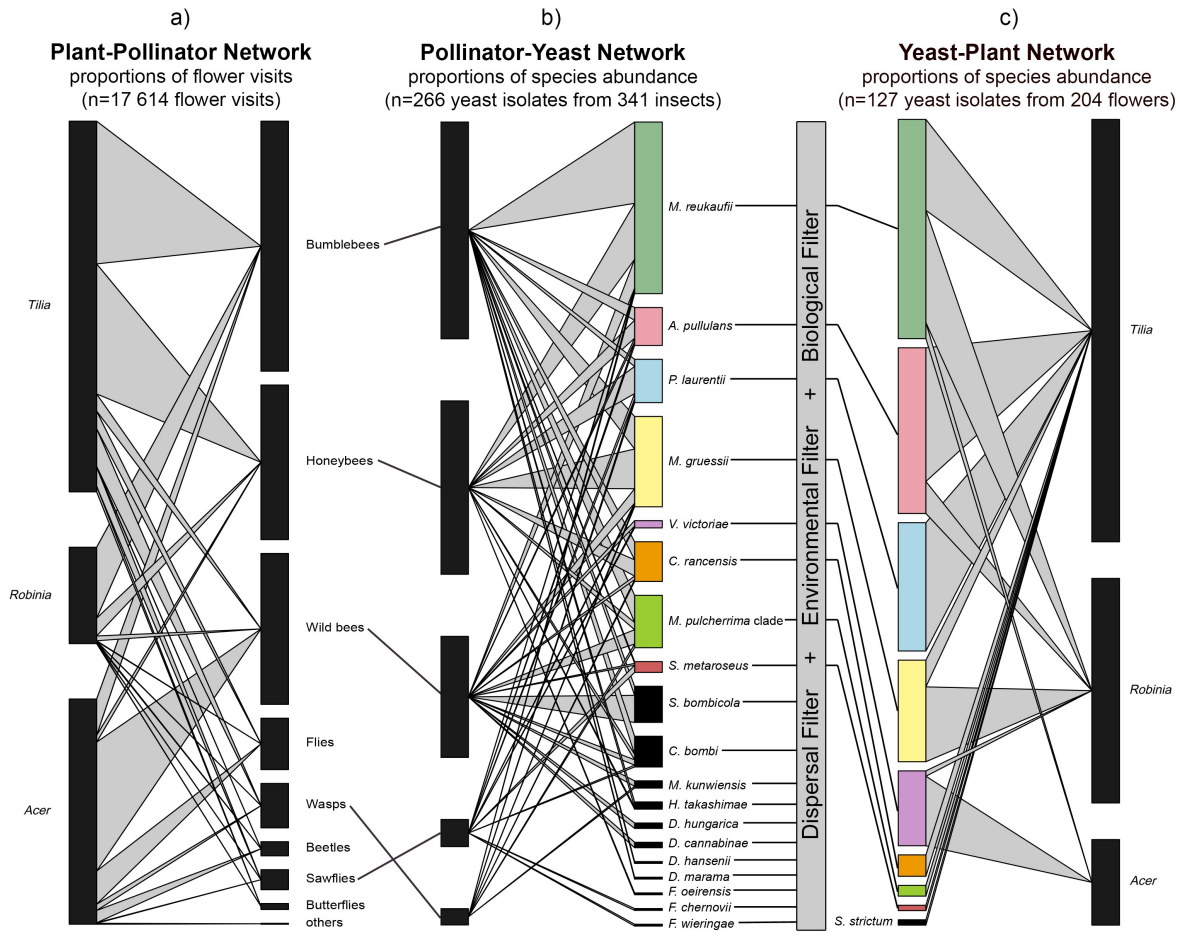

Figure 10: Bipartite networks of plant-pollinator interactions showing proportions of flower visits of nine insect groups on three tree species (a), pollinator-yeast-network showing proportions of 19 yeast species isolated from five insect groups (b) and yeast-plant-network showing proportions of nine yeast species isolated from floral nectar of three tree species (c). Colored yeast species in b) and c) were isolated both from pollinator and nectar samples, black colored species only from one kind of samples. Data from *Acer* were corrected for number of repetitions.

The yeast metacommunities from single tree individuals (Fig. 11a) together form a specific cluster per tree species. *Tilia* and *Robinia* have a big overlap in their yeast metacommunities, while *Acer* showed no overlap with another tree species. Within the tree species the dispersing yeast species pools from pollinators and the yeast metacommunities in flowers are overlapping for *Acer* and *Robinia*, but *Tilia* showed a strong separation between both groups.

In pooled metacommunities of nectar and pollinator groups per tree species (Fig. 11b) we note a high similarity between the dispersing yeast-species pools from bumblebees and honeybees to the yeast metacommunities from nectar for *Robinia* and *Tilia*. The dispersing yeast species pools of wasps and flies that only yielded valuable data for *Tilia* are less similar to the nectar-level than dispersing yeast-species pool from wild bees.

Nevertheless, the three wild bee-yeast-species pools are more similar to each other than to the nectar of their associated tree species.

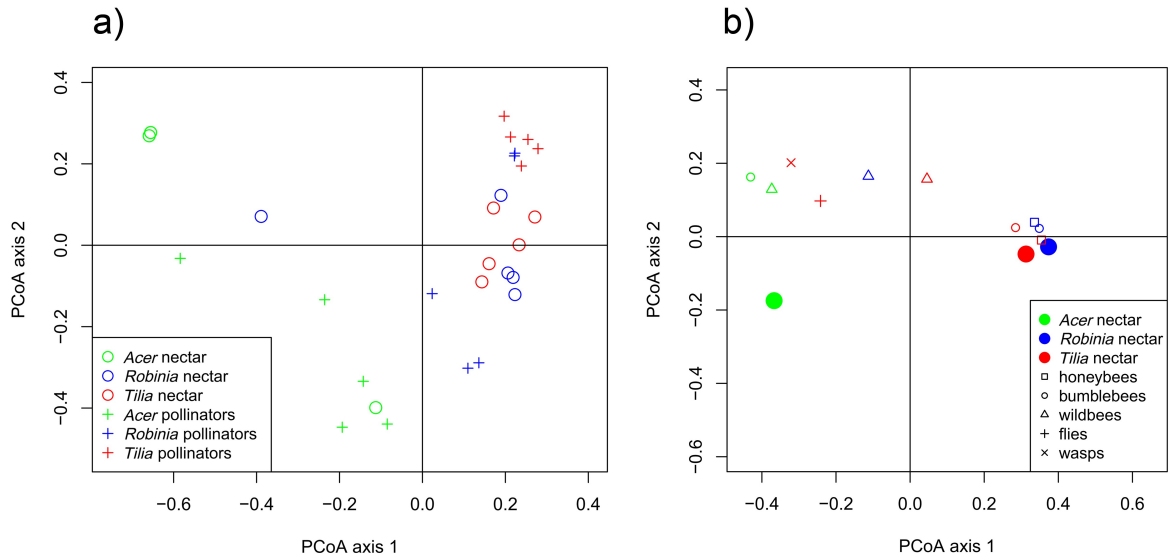

Figure 11: Similarities in yeast metacommunities visualized in PCoA plots using Bray-Curtis dissimilarity. a) Similarities of yeast metacommunities from pollinators and nectar of individual trees from three tree species. b) Similarities of the yeast species pools from different pollinator groups to the yeast-metacommunities in nectar pooled per tree species. Green points represent data from *Acer*, blue points from *Robinia* and red points from *Tilia*.

Associations of yeasts and pollinators

Most yeast isolates from pollinators belong to the group of nectar-specialists (59.1 %), the other 24.5 % and 16.4 % were transient yeasts and insect-associated yeasts (Fig 12). Compared to the proportions from the pollinator level, in the nectar the proportion of nectar-specialists decreased to 48.8 %, whereas transient species doubled to 49.6 % and insect-associated species almost vanished (1.6 %). Among the different pollinator groups, bumblebees and honeybees transported the most nectar-specialists (64.2 % and 64.6 %, Fig. 12), whereas sawflies transported only 13.3 %. Wild bees and wasps transported most insect-associated species with 27.0 % and 33.3 %, whereas honeybees and sawflies transported the least (7.3 % and 6.7 %). Most transient species were transported by sawflies with 80% and the least by bumblebees and wild bees with 15.6 % and 16.2 %.

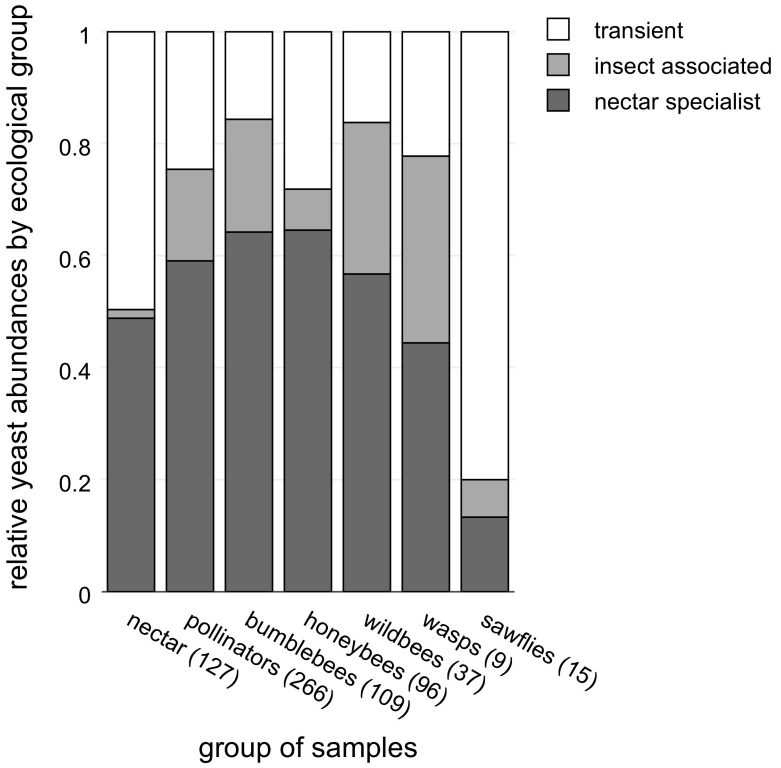

Figure 12: Proportions of yeast species classified by the ecological groups 'transient species', 'insect-associated' and 'nectar-specialist' of meta-communities of all nectar samples, all pollinator samples and single pollinator groups (bumblebees, honeybees, wild bees, wasps and sawflies). Numbers in brackets give the total number of yeast isolates for each bar.

Species indicator analysis (supplementary material A, appendix Table 15) revealed a probability of 52.6 % that bumblebees transport yeast species from the *M. pulcherrima* clade (p=0.027), and sawflies were indicated by *S. metaroseus* with a chance of 88.6 % (p=0.016). Three yeast species could not be assigned to as specific pollinator group but showed high probabilities to belong to the combined group of bumblebees, honeybees and wild bees: Probabilities were 92.3 % for *M. reukaufii* (p=0.001), 97.1 % for *M. gruessii* (p=0.001) and 92.5 % for *C. rancensis* (p=0.046), which are all nectar-specialists. Details on species indicator analysis for yeast-plant-interactions are given in supplementary material A, appendix Table 16.

2.5 Discussion

By studying yeast metacommunities from nectar and pollinators, our broad sampling allows for the first time to observe major changes in the composition of the yeast communities from pollinator to flower, indicating strong environmental and biological filter processes (Fig. 10 and 11). In addition, we support previous findings and

suggestions on species richness and dispersal. Our data on the entire pollinator spectrum extends results on species richness and abundance and on the effects of pollinator visitation frequency. All yeast species in nectar except one are linked to pollinators and are consequently present in the pollinator metacommunity. Insect-pollinators (Fig. 8, Brysch-Herzberg 2004; Herrera et al. 2010) transport more yeast species than are isolated from flowers, which was also shown for birds as pollinators (Belisle, Peay and Fukami 2012). Yeast species richness and abundance increase over the flowering season (Fig. 8, Glushakova, Kachalkin and Chernov 2014). Pollinator visitation frequency increases species richness and abundance of yeasts in flowers (Fig. 9, Vannette and Fukami 2017). Honeybees and bumblebees transported the most nectar-specialist yeast species (Fig. 12, Mittelbach et al. 2016).

Regional processes: dispersal with mobile linker community

The importance of regional processes on the structure of the metacommunity was indicated by dispersal frequency (measured as pollinator visitation frequency) and the similarity between metacommunities of single plant species and the dispersing species pool (as a subset of the regional species pool). Dispersal frequency was the main driver for species richness (Fig. 8a) and abundance (Fig. 8b) of the metacommunities, but species abundance was affected only by specific dispersal pathways (here: honeybees and bumblebees). Conversely, dispersal frequency did not explain the similarity in community composition between the locally established communities in flowers and the dispersing species pool (Fig. 11a). However, since a specific feature of the nectar yeast system is that dispersal and disturbance happen at the same time, we cannot disentangle these effects. Thus, the described effects of dispersal frequency can be explained by a higher disturbance frequency as well.

All of the nine yeast species found in nectar, except for *Sarocladiums strictum*, were also found on pollinator mouthparts, confirming pollinators as the main dispersal vector. In particular, together with results of Pozo et al. (2011), we identify bumblebees and honeybees as effective yeast dispersers. They dominated the pollinator community of *Robinia* (91 % of flower visits, supplementary material A, appendix Table 9) and *Tilia* (74 % of flower visits). On those tree species we found a high yeast species richness in the nectar (4 and 8 species, Table 3), and the proportion of pollinators that transported yeasts was very similar to the proportion of flowers that contained yeast (49 % to 44 %

for *Robinia* and 64 % to 60 % for *Tilia*, Table 3). The high yeast species richness in nectar of *Tilia* (8 species) might be caused by a very diverse pollinator community structure with around 5 % proportions each of wild bees, wasps, flies, and sawflies.

Wild bees and flies may have the lowest potential to disperse nectar yeasts. On *Acer*, we found only 2 yeast species in the nectar and observed a strong decline from the proportion of pollinators that transported yeast to the proportion of flowers that contained yeast (42 % to 20 %). This pattern could neither be explained by a small pollinator visitation frequency (supplementary material A, appendix Table 9) nor by a high nectar sugar concentration (Table 3); but it is most likely due to the composition of the pollinator community that consisted of mainly wild bees (50 %) and flies (18 %). The only previous study in which dispersal of nectar yeasts to a new flower was measured showed that even if yeast cells are attached to a honeybee proboscis dispersal to an artificial flower cup was successful in only half of the flower visits (Hausmann, Tietjen and Rillig 2017).

We found a large overlap in yeast communities between flowers and pollinators in social insects (honeybees and bumblebees) and a small overlap in solitary insects (Fig. 11b). Subsequently, most nectar-specialist yeast species were transported by honeybees and bumblebees (Fig. 12). Even if solitary wild bees show the same restriction to nectar and pollen as food source as their social relatives, their nesting substrate is very diverse ranging from soil, snail houses to wood or other plant materials. This may explain why they transport such a diverse yeast species pool different from the flowers they pollinate.

Most insect-associated yeast species were isolated from wasps and wild bees. Adult wasps hunt insects, which increases the chance to collect insect-associated yeasts. Sawflies transported the most transient yeast species, which they may have collected already during their larval development in soil, liquid manure or puddles. The composition of the established yeast metacommunities in floral nectar could be explained by the composition of the pollinator community and the typical yeast groups they transport. Taking together our results, yeast metacommunities in floral nectar may even be predicted quite well by analyzing the density of different pollinator groups.

Local processes

Of the 19 yeast species isolated from pollinators, 11 species were not found in the nectar, indicating a strong filtering process from pollinator to flower, as proposed by Herrera et al. (2010). A strong environmental filter effect was also shown by changes in the relative abundance of ecological yeast groups from pollinator to flower (Fig. 12). The relative abundances of nectar-specialists and transient species stayed almost stable from pollinator to flower, while that of insect-associated species declined. Also, specific ecological yeast groups are mainly associated with specific insect groups (Fig. 12).

The environmental filter effect was particularly evident for insect-associated yeast species (*Metschnikowia pulcherrima* clade, Fig. 12), most likely due to harsh environmental conditions in nectar. We measured nectar-sugar concentration as a local environmental variable and expected to find a lower yeast species richness with increasing sugar concentration due to a higher osmotic pressure, but we found the opposite (Table 3). This was most likely caused by a stronger effect of the species richness in the dispersing species pool that increased in the same direction as sugar concentration. Herrera et al. (2010) found a strong effect of the environmental filter of the nectar in *Helleborus foetidus* that leads to species-poor, phylogenetically clustered yeast communities. We detected many yeast species that arrive but cannot establish in the nectar habitat. Those species may be of interest because of their potential to establish when environmental conditions change in their favor, e.g. by rain or humidity.

The relative increase of transient yeast species (mainly *A. pullulans*, *P. laurentii* and *V. victoriae*) together with the small decrease of nectar-specialist yeasts (*C. rancensis*) from pollinator to flower (Fig. 12) shows that the transient species are competitive and can establish in the nectar environment. Most nectar-specialists like *M. reukaufii* and *M. gruessii* and the transient species *Sporobolomyces metaroseus* maintained almost stable proportions from pollinator to flower.

The biological filter could not be tested here, but strong competition effects for resources in nectar between different yeast species (Schaeffer, Vannette and Irwin 2015) and between yeasts and bacteria (Canto et al. 2008; Herrera, Garcia and Pérez 2008; Vannette and Fukami 2018) have frequently been reported. If nectar yeast species arrive in a flower at the same time they almost always coexist over the lifetime of the

flower, even if some species show faster growth rates than others (Tucker and Fukami 2014). Competitive exclusion or growth suppression was almost only observed if two species arrive at different times with advantages for the first arriving species. Such priority effects can be strong determinants of community composition within single flowers (Tucker and Fukami 2014).

Comparison with other systems

Nectar yeasts transported by pollinators and those isolated from floral nectar are the source for each other. However, a single pollinator can use several flowers as a resource, including flower types not included in our sampling. Accordingly, we observed a higher species richness and abundance of yeast transported by pollinators than yeasts isolated from flowers. This (partially) circular species transfer and the yeasts' ability to change nectar chemistry and scent and thus influencing pollinator behavior (Herrera, Pozo and Medrano 2013; Schaeffer and Irwin 2014; Rering et al. 2017) makes the nectar system fairly unusual (e.g. in contrast to islands where species invasion is mostly unidirectional). Nevertheless, there are many similarities to other systems.

The flower (or nectar droplet) represents an 'island'-type ecosystem, highly dynamic in space and time, and surrounded by an unsuitable matrix. Similar conditions can be found in temporary ponds. Already 50 years ago the importance of Odonata (Maguire 1963), Diptera (Revill, Stewart and Schlichting 1967), Coleoptera (Schlichting and Milliger 1968) and Hemiptera (Schlichting, and Sides 1969) for the dispersal of aquatic algae and protozoa was shown. Five times more algae and protozoa genera were found in ponds than transported by Hemiptera, but not all of these genera only depended on dispersal by insects (Schlichting and Sides 1969). In contrast, our data show double the yeast species richness transported by pollinators than what is established in flowers. Much literature is available about passive bird-mediated dispersal of propagules of aquatic invertebrates and plants. A higher density of birds does not necessarily lead to a higher species richness of aquatic invertebrates in ponds (Green and Figuerola 2005), as we have observed here for insects and nectar yeasts. We assume our pattern in the nectar system is caused by shorter habitat lifetime and a much higher number of (uninvaded) flowers-habitats for yeasts, leading to quite high colonization probability for rare species or weak competitors. The effect of environmental filtering in nectar yeasts is likely not as complex as for ponds that are much larger and offer more habitat

types and structures that allow species to avoid direct competition. Still, priority effects as part of the biological filter are equally important for zooplankton in ponds (Meester 2007) as for the nectar-yeast system (Tucker and Fukami 2014).

Conclusion

Our results show that the dispersal frequency of the mobile linker community plays an important role in determining the species richness and the overall species abundance of the metacommunity, whereas local processes like environmental filtering effects mainly shape the species composition. We recommend the nectar-yeast system with our new approach of identifying the insect-migrating dispersing species pool as a promising system for further studies on dispersal and colonization processes. Considering movement ecology and flower-animal interactions allows new insights into the role of ecological filters during colonization processes and therefore a better understanding of the resulting metacommunity.

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3 CHAPTER 3: NECTAR YEAST COMPETITORS, BUT NOT NECTAR YEASTS, OVERWINTER WITH BEES

3.1 Abstract

The lifecycle of yeasts specializing on nectar, *M. reukaufii*, *M. gruessii* and *C. rancensis*, is still not fully understood. We do not know how and where they survive the winter to then colonize nectar of flowers in spring. A common hypothesis is that the nectar yeasts overwinter with pollinating insects. Here, we analyze bees and their food reserves after hibernation before they leave their nests. We do this for bees from three groups with different social organizations and hibernation strategies. Although we analyzed 131 insects and 190 insect-related samples (stored food or excrements), we have not found any isolate of specialized nectar yeast species after winter. However, we found six fungi and yeast species which are known to colonize nectar but are not only restricted to it. Two species, *Aureobasidium pullulans* and *Debaryomyces hansenii*, occur in very high abundance in floral nectar and are strong competitors with nectar yeasts in floral nectar. Thus, the question where nectar yeasts overwinter remains open, but we can exclude bees as main vector. Instead, we found that many transient yeast species that are frequently found in nectar and act as nectar yeast competitors, mainly *A. pullulans* and *D. hansenii*, can use bees to overwinter.

3.2 Introduction

Nectar yeast and bacteria have become an important topic in community ecology, playing an influential role in pollination (Schaeffer et al. 2017), bee health (Graystock, Goulson and Hughes 2015), and plant fertility (Herrera, Pozo and Medrano 2013). Microbial community structures and physiological footprints in nectar have been studied especially in yeasts, particularly using highly specialized and ecologically unique organisms from the ascomycetous genus Metschnikowiaceae, mainly *Metschnikowia reukaufii*, *Metschnikowia gruessii* and *Candida rancensis* (Lachance 2016). Found in no other habitats, these apparently specifically adapted species have been only isolated from pollinating insects, birds and floral nectar, where they can be very abundant and dominant (e.g. Pozo, Lachance and Herrera 2012; Mittelbach et al. 2015). Pollinators act as vectors for these yeasts, enabling their survival in this temporally and spatially fragmented habitat through transport and inoculation into new flowers (Brysch-

Herzberg 2004). We do not know about further life-stages or -strategies of these yeast species, especially about their survival outside the flowering season and during winter. Overwintering with hibernating social insects has been proposed (Brysch-Herzberg 2004), but this hypothesis has not yet been tested.

Floral yeasts need to enter the pollination niche in spring. Due to their fast proliferation and high dispersal frequency, few entrance events should be sufficient to initiate and maintain a population in nectar. Yeasts then maintain their populations in the floral system and at some unknown point have to reach a suitable habitat to survive the winter. An introduction and overwintering of nectar yeasts in buds has not been shown, but cannot be completely excluded, because previous studies did no systematic research and sampled mainly late flowering plants, when overwintering buds of early flowering plants would be relevant. Moreover, the fact that nectar yeasts were never observed as endophytes in culture-based studies (Fonseca and Inácio 2006), reduces the likelihood that they establish from floral tissues. As far as soil is concerned, several studies on yeasts in soil never found these species in different land use types in Germany (Yurkov, Kemler and Begerow 2012) and Slovakia (Sláviková and Vadkertiová 2000, 2002, 2003).

Yeast species can proliferate to high cell densities in nectar and at least partly attract pollinators (Herrera, Pozo and Medrano 2013; Schaeffer and Irwin 2014). Consequently, cells are up-taken by foraging bees in large amounts. Thus, it is reasonable to hypothesize that yeasts use pollinators, their nesting site or food reserves as overwintering sites, as proposed by Brysch-Herzberg (2004). Bees as the main pollinators in Europe (Williams, Corbet and Osborne 1991) disperse a large proportion of nectar yeast populations. But bees can have very different social organization and hibernation strategies. Three of those strategies and the resulting conditions for overwintering yeasts are introduced below: solitary bees nesting in wood, bumblebees with a social lifestyle and queen-only hibernation, and honeybees with social lifestyle and hibernation of the entire colony.

In bumblebees, the queens hibernate individually outside their colony in loose soil, litter, under bark, stones or cavities in wood, where they withstand temperatures down to the freezing point, but only rarely below (Yoon et al. 2013). Bumblebee queens do not consume food during hibernation at all. The leftover food in abandoned bumblebee soil-nesting colonies, which is a mixture from pollen and nectar, is deep enough below soil

surface to keep temperatures above the freezing point. In some bumblebee species that nest aboveground, temperature in winter can be lower.

Wild bees hibernate as diapausing larvaes or pupaes (cocoons) in single chambers in soil, wood or other cavities provisioned with a mixture from nectar and pollen, which is consumed by the development stages or early imago stage of the bees. The species *Osmia cornuta* and *Osmia rufa* that nest in holes in wood survive temperatures down to -30°C (Krunić and Stanisavljević 2006) through the production of glycerol in amounts constituting 1.8% of fresh bodyweight (Krunić, Milovanović and Radović 1976).

Worker honeybees endure the whole winter period as adults. They organize themselves into a tight cluster during winter and produce temperatures between 12 and 30°C by muscle activity. Temperature in stored food equals the temperature of bee bodies but can be lower outside the bee cluster. Inside the hive the temperature is at least 8–9°C higher than the outside air temperature (Stabentheiner et al. 2003), but can drop below 0°C. Although, temperatures on honeybee body surface as well as in stored food can reach optimal growth temperature of nectar yeasts around 25°C (Pozo, Lachance and Herrera 2012; Tucker and Fukami 2014), other conditions make it difficult for yeasts to survive. Only Honeybees cover all surfaces of the hive with propolis – a mixture of resin from buds, which has strong antimicrobial and antifungal properties (Kujumgiev et al. 1999; Ota et al. 2001). During winter honeybees feed each other with honey, which sugar concentration and osmotic pressure exceeds the survival tolerance of any of the nectar yeasts species, which can barely tolerate over 50% sugar concentration (Herrera, Pozo and Bazaga 2012; Pozo, Lachance and Herrera 2012). Therefore, a survival of yeasts on honeybee's proboscis or in stored honey is unlikely. In addition, stored pollen (which is always mixed with nectar), which may provide a highly concentrated source of amino acids and carbohydrates (Serra Bonvehí and Escolà Jordà 1997), is hygroscopic, decreasing the chance that the medium falls dry. That effect may draw water from yeast cells and increase osmotic pressure, too. On the other hand, in overwintering honeybee hives water evaporates from the bee's metabolic activity and condenses on colder combs. This water can dilute provisions and may reduce osmotic pressure again.

We do not know the lowest temperatures nectar yeasts tolerate for survival. Although low temperature in wild bee nesting sites decreases competition with other temperature sensitive bacteria and fungi, we expect the higher temperatures in honeybees and

bumblebees make it more likely for nectar yeast to overwinter. Nectar-yeast survival (and growth) in pure honey samples is generally unlikely, since osmotic pressure is too high. Previous studies never found nectar yeasts in pure pollen (De-Melo, Estevinho and Almeida-Muradian 2015) or pure honey samples (Snowdon and Cliverb 1996), but *M. reukaufii* was isolated from stored bee-bread (fermented mixture of pollen and nectar) from honeybees (Babjeva and Gorin 1973; Batra, Batra and Bohart 1973) and the solitary bee species *Megachile rotundata*, *Nomia melanderi*, *Anthophora pacifica* and *Ptiloglossa spec.*, which are representatives of wood-nesting and soil-nesting bees (Batra, Batra and Bohart 1973). Also, bumblebees store bee-bread, but to our knowledge it was not yet tested for the presence of nectar yeasts. 85 % of the fermenting yeasts in bee-bread belong to the genera *Torulopsis*, *Candida* and *Cryptococcus* (Gilliam 1979a). Beside yeasts the majority of molds identified in bee-bread were Penicillia, Mucorales, and Aspergilli (Gilliam, Prest and Lorenz 1989). Furthermore, bee-bread is colonized by lactic acid bacteria, which are added with bees' digestive fluids (Gilliam 1979b). However, the *M. reukauffi*-containing bee-bread samples mentioned above were taken in summer or during overwintering, but not after overwintering, when bee-bread is usually consumed by bees and leftovers are only available in honeybee colonies.

In order to survive unsuitable conditions up to a 6-month period, yeast cells would need to form resting stages or even spores. Although all nectar specialist yeasts we consider here are capable to form chlamydospores under culture conditions (Kurtzman, Fell and Boekhout 2011), information on their occurrence in natural habitats, as well as data on formation, development conditions and stress resistance are scarce. In *M. reukaufii*, spore formation is preceded by the differentiation of diploid cells into chlamydospores, which are characterized by a thickened wall and one or more fatty globules, sometimes called 'reukaufii cells' (Lachance 2016). Whether Metschnikowia chlamydospores are capable of dormancy or stress resistance and if they play a role in overwintering has not yet been investigated. Conditions for their development vary from culture to culture (Pitt and Miller 1968). Glushakova et al. (2015) tested drought resistance of yeasts and found *M. reukaufii* dead within one month under warm and dry conditions, without checking if cells formed chlamydospores or other resistant stages under the given conditions. Survival of nectar yeasts under cold and wet conditions, as they may occur during overwintering with bees, were not yet tested.

The aim of the present study is to test if nectar yeasts overwinter with hibernating bee individuals or in their nesting sites. We isolated yeasts from bees and their stored food directly after hibernation, covering three representative bee groups: wood-nesting solitary bees, bumblebees (*Bombus terrestris*), and honeybees (*Apis mellifera*). After consideration of the different hibernation conditions like temperature, osmotolerance and bee lifestyle we hypothesize it to be most likely for nectar yeasts to overwinter with bumblebee queens or in stored bee-bread in bumblebee nests (belowground) or honeybee colonies.

3.3 Materials and Methods

Sampling yeasts from wild bees

Ten artificial wooden nesting units for solitary wild bees were placed at five locations on April 25th 2016 (two per site) in the agriculturally used Quillow catchment, Brandenburg, Nordwestuckermark, Germany. Each nesting unit was made from 4 standardized (beech-)wooden plates (8 x 11 x 2.5 cm) with squared holes of 3, 4, 6 and 8 mm diameter and a wooden top plate to protect the unit from rain (Fig. 13a). Each unit was attached to a pole of 1 m height facing southwards, next to a hedgerow or a group of shrubs.

Nesting units were collected on October 28th 2016, placed in a plastic box with holes for air exchange and stored outside, protected from wind and rain. On February 15th 2017, each unit was placed into a closed insect net (1 mm mesh size). Every day we checked for hatching insects. Seven bees hatched in March, seven in April and 29 bees and 5 wasps in May 2017. Each hatching insect was placed into a single glass tube and killed with CO_2 gas. Before bees were pinned, their probosci were removed with sterile forceps under a dissecting microscope. The probosci were mixed with 50µl sterile water in 200µl PCR-tubes (Eppendorf, Hamburg, Germany). In the evening of the sampling day, first each bee was rolled over a 1% sugar agar plate (0.3 % yeast-extract, 0.3 % malt-extract, 0.5 % peptone, 1.0 % dextrose, 2.0 % g agar, 95.9 % water, values in % w/w) several times, then the probosci sample of the bee with the water were streaked on the same agar plate. Samples were incubated in the dark at 25°C for five days.

At end of June, after no further bees had hatched for over two months, nesting units were opened and excrements from abandoned chambers were taken. The excrement samples were mixed with 50 µl sterile water and processed just like the bee samples.

To check if nesting units already contained yeasts before installation, two nesting holes from each of the 10 nesting units (a total of 20 samples) were flushed with water. This water was streaked on agar. No vital yeasts were found in the nesting units before the experiment started.

Bees were determined using common keys: Schmid-Egger and Scheuchl (1997); Amiet, Müller and Neumeyer (1999); Scheuchl (2000, 2006); Amiet et al. (2001). The nomenclature of wild bees follows Westrich and Dathe (1997). Wasps (Specidae and Sapygidae) were determined with Dollfuss (1991) and Witt (2009). Capturing and killing of bees and wasps is prohibited (§ 44 (1) Nr. 1 Federal Nature Conservation Act Germany – FNCA). According § 45 (7) Nr. 3 FNCA a special permission was granted by the Regional Department for Environment Brandenburg to collect bees and wasps for this research.

Sampling yeasts from honeybees

Eight beekeepers in the city area of Berlin contributed to the sampling in this study. Four of them harvested honey and fed the bees with sugar water as winter food from August to October 2016. The other four beekeepers did not feed sugar water to the bees but left them as much honey as they needed for the winter. Of each beekeeper, we chose one beehive randomly for sampling. Sampling was done from February 10th to 20th 2017 when temperatures were still too cold for bees to fly out. Bees might have had their first defecation flight already, but typically this is not combined with foraging. Therefore, we expect the bees to have had no contact with relevant yeast sources in the new season. From each hive, we collected 10 samples each from honey, bee-bread (pollen-nectar mixture) and worker bees. Honeybees were placed individually into glass tubes and processed as described above. Honey and pollen were taken with a sterile toothpick from single wax cells, mixed with 50 µl sterile water and processed as described before.

Sampling yeasts from bumblebees

Two bumblebee colonies (Natupol standard, Koppert Deutschland GmbH, Straelen, Germany) were placed each into one of two one-side-open flight nets (2 x 2 x 2 m, mesh

size 0.8 mm, FA.BIO 02, Hartmann-Brockhaus, Pfaffenhofen-Wagenhofen, Germany, Fig. 13b) on June 27th 2017; these colonies started to produce queens in the middle of August. Flight nets were closed in the middle of August. Soil inside the netted area was loosened with a spade to allow bee queens to dig for hibernation. When nets were closed, we started to feed the bees inside the nets with artificial nectar (AN) solution (25% sucrose, 0.32mM Casein digested amino acids) containing cells of the nectar yeast *M. reukaufii* in a concentration of 20 000 cells per µL. The used strain is deposited at Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures under accession number 'DSM100740'. Solutions were presented in two cups with each 2.5mL. The cups were placed in a container filled with water, to deny access to ants and prevent other animals from feeding. Solutions were replaced approx. every 3rd day. New solutions were mixed from a constantly growing stock of *M. reukaufii* in 12mL of artificial nectar (25% sucrose w/v solution supplemented with 0.32mM amino acids from casein hydroxilate), stored at 25°C. The cell concentration of 20 000 cell per µl is related to an OD value of 0.15 measured at 660 nm in 100µl in a 96-well plate (measured with a Benchmark Plus microplate spectrophotometer, Bio-Rad Laboratories GmbH, Munich, Germany).

Bumblebee nests were removed on March 1st 2018. Bombus terrestris stores food in small pots made from wax. From each nest we randomly chose ten of those food pots and leftover food was scratched off from the pots with a sterile toothpick. The material was processed as described for wild bee food. Three bumblebee queens hatched on April 4th 2018 from the soil in one net, were caught directly and were processed as described before. In the other of the two nets no bumblebee queen hatched at all.

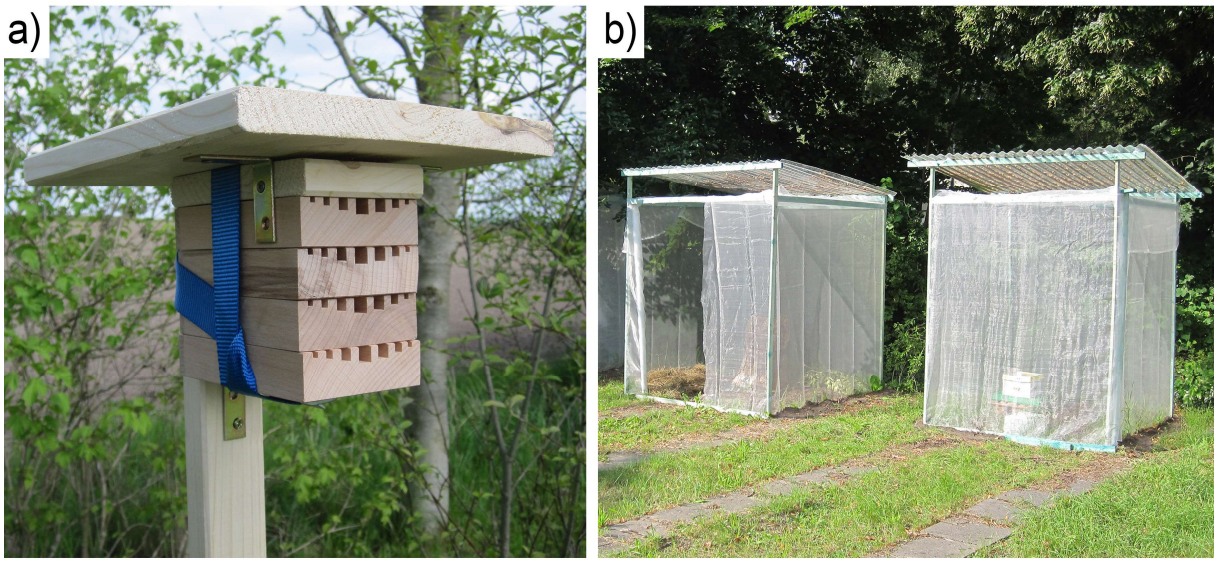

Figure 13: a) Nesting unit for solitary wild bees. b) flight nets of bumblebee colonies.

Molecular identification of yeast cultures

All different yeast morphotypes from incubated agar plate samples were transferred to single agar plates for isolation and grown again for 5 days at 25°C in the dark. For molecular identification based on the D1/D2 domain of the large subunit nuclear ribosomal RNA, cell material was taken from an agar plate and added to a 50 µl Master Mix made of 10 µl Firepol Master Mix (Solis Biodyne, Tartu, Estonia), 36 µl water, 2 µl primer NL1 (5′-GCA TATCAA TAA GCG GAG GAA AAG-3′) and 2 µl primer NL4 (5′-GGT CCG TGT TTC AAG ACGG-3′). The PCR temperature profile consisted of an initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 55°C for 10 s and 72°C for 20 s, and a final extension at 72°C for 1 min. PCR products were examined by agarose gel electrophoresis and quantified using a Nano Photometer (Implen, München, Germany). Afterwards we purified the PCR products using a PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Obtained amplicons were sequenced by LGC Genomics (Berlin, Germany) using the same reverse primers as those used for amplification. Single sequences were identified with Mycobank database (Robert, Stegehuis and Stalpers 2005) using pairwise sequence alignment. Species identification was accepted if similarity was 99% or more. Yeast systematics follows Liu et al. (2015) and Wang et al. (2015).

3.4 Results

Nesting site

48 insects hatched from the nesting sites: 43 bees from 4 species and 5 wasps from 2 species. Only 13 isolates of yeasts and yeast like fungi were isolated from the 43 bees (see Table 5). The five wasp samples did not contain any yeasts. Most abundant was the yeast-like ascomycetous fungi species *Aureobasidium pullulans*, which was isolated from 11 out of 43 bees (25.6% of samples) from 3 different bee species. The yeast species *Curvibasidium cygneicollum* and *Filobasidium wieringae* were each isolated only once from males of the bee species *O. bicornis*. No yeast isolates were obtained from 10 excrement samples from nesting chambers of *O. bicornis*. In March and April, respectively, 7 male bees hatched. In May 24 female and 5 male bees hatched. Eight yeast isolates came from male bees hatched in March. No isolates were found from bees hatched in April and 5 isolates came from females hatched in May.

Honeybees

Sugar water and honey from honeybee colonies both contained *Debaryomyces hansenii* and *Candida magnolia* (see Table 5). *D. hansenii* was more frequent in honey (40% of samples) than in sugar water (5% of samples). A similar pattern was found for yeast isolates from bees, which contained more *D. hansenii* in honey-fed colonies (28% of samples) than in sugar-fed colonies (10% of samples). *C. magnolia* was more frequent on bees from sugar-fed colonies (28% of samples) than honey-fed colonies (5% of samples). In addition, *Zygosacharomyces rouxii* was only found 3 times (8% of samples) on bees from honey-fed colonies, but not from sugar-fed colonies. Bee-bread from sugar-fed colonies did not contained any yeasts, but bee-bread from honey-fed colonies contained all three yeast species (*D. hansenii*, *C. magnolia*, and *Z. rouxii*), with *D. hansenii* being found in 18% of samples.

Bumblebees

Only three bumblebee queens from one of two colonies hatched on April 4th 2018. From all of them we isolated *D. hansenii*, but no other yeast species (see Table 5). From the 20 bee-bread samples from bumblebee nests we isolated 7 times *D. hansenii* (35% of samples) and 20 times *Z. rouxii*, which was present in all samples. Even if we fed the bumblebee colonies with *M. reukaufii* in late summer we could not isolate it after winter.

Table 5: Number of samples, yeast isolates and yeast species from solitary insects from nesting sites (n=10), honeybee colonies (n=8) and bumblebee colonies (n=2). Four honeybee colonies were fed with sugar water before winter and four were not. f=female, m=male.

Material	# samples	# isolates	*A. pullulans*	*C. cygneicollum*	*F. wieringae*	*D. hansenii*	*C. magnoliae*	*Z. rouxiii*
Nesting site								
Wild bees total	**43**	**13**	**11**	**1**	**1**	**-**	**-**	**-**
Hylaeus communis	2(1f,1m)	-	-	-	-	-	-	-
Hylaeus punctatus	10 (3f,7m)	1	1(1f)	-	-	-	-	-
Megachile centuncularis	3(3f)	1	1(1f)	-	-	-	-	-
Osmia bicornis	28	11	9(3f,6m)	1(1m)	1(1m)	-	-	-
Bee excrements	10	-	-	-	-	-	-	-
Wasps total	**5(5m)**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Passaloecus spec.	3(3m)	-	-	-	-	-	-	-
Sapygus spec.	2(2m)	-	-	-	-	-	-	-
Honeybees (sugar-fed)								
Sugar water	40	12	-	-	-	2	5	-
Bee-bread	40	12	-	-	-	-	-	-
Bee	40	13	-	-	-	4	11	3
Honeybees (honey-fed)								
Honey	40	7	-	-	-	16	2	-
Bee-bread	40	0	-	-	-	7	2	3
Bee	40	16	-	-	-	11	2	-
Bumblebees								
Bee	3	3	-	-	-	3	-	-
Bee-bread	20	27	-	-	-	7	-	20

3.5 Discussion

We did not find vital cells of nectar-borne yeast species on the body surface or the stored food of solitary bees in wood, bumblebees and honeybees. Instead, we found solitary bees as a hibernation vector of mainly *A. pullulans* and less often two yeast species only rarely found in nectar (Table 5). Bumblebees and honeybees show a high overlap in overwintering transient yeast species over all samples; in honeybees *D. hansenii* was more frequent and in bumblebees *Z. rouxii*.

The two most abundant overwintering fungi and yeast species *A. pullulans* from solitary bees and *D. hansenii* from bumblebees and honeybees appear in nectar as transient

species with occasionally very high abundances. Glushakova, Kachalkin and Chernov (2014) describes *D. hansenii* as one of two predominant and early-occurring yeast species in floral nectar in spring in the Moscow region and Pozo, Lachance and Herrera (2012) identified the species as one of the five species with the highest growth rates in nectar of two plants in Spain. *A. pullulans* is a yeast-like filamentous ascomycetous fungus that is frequently found in nectar, but based on its physiology and ecological niche it is not considered a specialist in nectar. *A. pullulans* showed competitive growth rates in artificial nectar compared to nectar yeasts (Vannette and Fukami 2016) and several studies that isolate yeasts from floral nectar report *A. pullulans* as one of the most frequently found species (De Vega and Herrera 2012; Vannette and Fukami 2017; Wehner et al. 2017), the most abundant species (Pozo, Herrera and Bazaga 2011) or even as omnipresent species (Pozo, Lachance and Herrera 2012). Although *Z. rouxii* was omnipresent in abandoned bumblebee hives after winter, it is only rarely reported from nectar (Pozo, Lachance and Herrera 2012; Glushakova, Kachalkin and Chernov 2014; Wehner et al. 2017).

Yeast overwintering with wood-nesting solitary bees

We analyzed bees of four species that nest in wooden holes and found three yeast species that are known to colonize nectar. The most abundant species *A. pullulans* is very abundant in nectar, while two other yeast species are only rarely found in nectar (*F. wieringae*: Vannette and Fukami 2016; Wehner et al. 2017; *C. cygneicollum*: Mittelbach et al. 2015) and are attributed to other plant related substrates.

It is not surprising that we did not find nectar yeasts from hatching solitary bees, because these individuals never had direct contact to floral nectar and its microorganisms. Its only chance of contact with nectar-borne yeasts is through the pollen-nectar mixture collected by the mother during the previous flowering season. Since it is not likely that floral yeasts can survive digestion by the bee larvae (we did not find any yeast in leftover fecal material), leftover yeast cells would need to stick to the body surfaces of the imago before hatching. Temperatures in wild bee nesting sites can potentially go down to -30°C (Krunić and Stanisavljević 2006). It is notable that the two yeast species *C. cygneicollum* and *F. wieringae* survived such harsh conditions.

The wasps *Passaloecus spec*. and *Sapygus spec*. are known to hunt larvae of other insects, but not to collect pollen and nectar for their brood. Thus, we did not expect to find any nectar yeasts there.

Yeast overwintering with bumblebees

We sampled three bumblebee queens and identified only *D. hansenii*. This complements findings of Brysch-Herzberg (2004) from six bumblebee queens after hibernation. Although Brysch-Herzberg (2004) did not find *D. hanseni*, he isolated related *D. marasmus* and additionally *Metschnikowia kunwiensis* and *Z. rouxii*. We found only two transient yeast species in the stored bee-bread from abandoned bumblebee colonies (*D. hansenii* and *Z. rouxii*). Even if yeasts would not stay vital on the body surface of a bumblebee queen, bumblebees could collect those yeasts in the early new season when they inspect abandoned colonies or even start a new colony in a foreign abandoned one (Pomeroy 1981; Donovan and Wier 1978; Barron, Wratten and Donovan 2000). Since we did not find nectar yeasts in the abandoned bumblebee colonies, this behavior favors dispersal of the found nectar yeast competitors.

Yeast overwintering with honeybees

We found only three yeast species in the honeybee colonies; these differed in abundance between colonies fed with sugar water and those fed their own honey before winter. Bee-bread from honey-fed colonies contained three yeast species, whereas bee-bread from sugar-fed colonies contained no yeasts at all. This leads to the conclusion that the exchange of honey with sugar interrupts the transport of yeasts to the pollen storage. *D. hansenii* was more abundant in honey and on bees from honey-fed colonies, while *C. magnoliae* was better supported in sugar-fed colonies. The presence of *Z. rouxii* did not show a clear pattern. *C. magnoliae*, apart from the two other ubiquitous species, is known to be associated with bees (Rosa et al. 2003) and is regularly reported from nectar (Herrera, Pozo and Bazaga 2012; Misra et al. 2012; Álvarez-Pérez and Herrera 2013).

Nectar yeast overwintering unsolved

Although in late summer up to one third of pollinators (own observations in 2016) and up to 71 % of bumblebees (Brysch-Herzberg 2004) transport nectar yeasts on the proboscis we could not find a single isolate of the nectar-specialized yeasts in our

comprehensive sampling after hibernation of three bee groups in early spring. We know that at least for bumblebees, *M. reukaufii* was introduced to the sampled nest, because we fed the colonies in late summer with this nectar yeast species. Furthermore, in honeybees and solitary bees the introduction of *M. reukauffi* to stored bee-bread before winter was reported in previous studies (Babjeva and Gorin 1973; Batra, Batra and Bohart 1973). Nectar yeasts are introduced into nesting sites of bees and due to their physiology should become part of the microbial community in stored food. However, these yeasts cannot survive the conditions in this substrate or on the bee body during the winter period, possibly because of limited nutrient sources, low temperatures, high osmotic pressure, the presence of antifungal substances like propolis, or competition with bacteria and fungi. Still, because of fast exponential growth and fast dispersal of nectar yeasts with pollinators only few inoculation events into flowers could theoretically be sufficient to subsequently establish abundant populations (Hausmann, Tietjen and Rillig 2017). Consequently, successful survival and re-distribution of nectar-borne yeast cells might be strongly selective, allowing only a small number of haplotypes to start new communities. Thus, perhaps the sample sizes in this study were too small to find that single nectar-yeast-carrying insect individual. Despite this, we think all the hibernation strategies with bees we tested here would have a very low chance to allow reliable nectar yeast hibernation.

Brysch-Herzberg (2004) already suggested that, in agreement with (Babjeva and Gorin 1973), insects other than bumblebees are the over-wintering sites of nectar yeasts. We have shown that those are not honeybees and not wood-nesting solitary bees. We think that ants could be a possible nectar yeast hibernation site, because of several reported yeast isolations from ants (Golubev and Bab´eva 1972; Middelhoven et al. 2003), their behavior to forage nectar and their ability to carry nectar yeasts (De Vega and Herrera 2012). Ants can even grow fungi for their own nutrition (Weber 2012) and store food in winter. Another possibility would be hibernation with solitary bees nesting in soil. We sampled only 4 of 383 species of solitary bees in the sampling region of Brandenburg, Germany (Saure and Dathe 2000) and only species that are nesting in wooden wholes, which is only one third of all solitary bee species. We believe the chance to find nectar specialist yeasts when analyzing yeasts from other solitary bee species is high because of very diverse nesting and hatching strategies, especially from the larger group of soil nesting species. Additionally, soil temperature rarely drop below 0°C if bees dig deep

enough, which increases the chance for successful yeast hibernation. On the other hand, hibernation conditions with soil nesting solitary bees should be like those of soil hibernating bumblebee queens and in our study these did not contained nectar yeasts.

Conclusion

Following discussion of conditions for hibernation of yeasts with different bee groups, we suggested the highest potential for yeast hibernation on bodies of bumblebee queens and in stored bee-bread in bumblebee nests and honeybee colonies; nevertheless, we could not isolate any vital nectar-specialized yeast species from the investigated wild bees (wood-nesting), honeybees, bumblebees or their stored food. A pathway that was not analyzed here but seems to have a high chance for hibernating nectar yeasts are ants and soil nesting solitary bees. The question of nectar yeast overwintering thus remains unsolved, but we can exclude honeybees, bumblebees and wood nesting solitary bees as a reliable hibernation site for nectar yeasts.

We showed that some transient yeast species can use bees as hibernation sites. Some of those species, like *A. pullulans* and *D. hansenii*, are frequently and in high abundances found in nectar, some are only rarely reported in nectar, such as *F. wieringae*, *C. cygneicollum*, *C. magnoliae* and *Z. rouxii*. For these yeast species, bees are definitely a reliable hibernation pathway even if those yeasts can use other pathways as well. So, even though it has been expected that nectar-borne-yeasts hibernate with bees, we found no evidence to support this, but instead bees support hibernation of transient yeast species, subsequently competing with specialized nectar yeasts in flowers.

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GENERAL DISCUSSION

The results of this thesis contribute to our understanding about how pollinator foraging affects the ecology of nectar yeasts in local habitats and at the metacommunity level. While pollinators are needed for the passive dispersal of nectar yeasts, the extraction of nectar by pollinators always reduces the local nectar yeast population density. When the pollination frequency is too high, the local population can strongly decline resulting in cell densities too low for efficient dispersal. On the other hand, when the pollination frequency is too low, high population growth can occur in flowers but dispersal events to new flowers are strongly decreased (Chapter 1). On the metacommunity level, the identity of the pollinator specifies which group of yeasts is dispersed and how efficiently cells are dispersed. The frequency of the pollinators determines overall yeast abundance and species richness in flowers (Chapter 2). We did not find evidence that bees have beneficial effects on yeasts during overwintering. Instead we found bees could have negative effects on nectar yeasts, by offering overwintering habitats for fungi and yeast species that act as strong competitors for nectar-yeasts in flowers (Chapter 3). Together, these findings not only strengthen the perspective that nectar yeasts depend on pollinators for dispersal, but that many aspects of their ecology are controlled top-down by pollinator foraging. This perspective will be discussed in more detail in the next paragraphs. Finally, we will discuss the bottom-up effect of yeasts on pollinators – i.e. how nectar yeasts can change pollinator decisions.

Top-down effects of pollinators on nectar yeasts

Exponential growth is not enough (Chapter 1)

In the first chapter, we quantified nectar yeast cell numbers on pollinator mouthparts and in artificial flowers at different steps of dispersal, and model local nectar yeast population dynamics. The model revealed how nectar yeasts can develop cell densities with ecological effects. First, the pollination frequency must be in a beneficial range: high enough to ensure yeast cell inoculation, but low enough to keep decimation events (reduction of yeast population with nectar foraging) small. Second, yeasts must have a beneficial combination of traits that balance growth rate and remaining fraction. If both processes can be performed at least at an intermediate level the system can develop cell densities with ecological effects. Thus, nectar yeast populations not only need a high

growth rate to compensate high frequent disturbance events but need other physiological traits or adaptions in behavior that ensure a high remaining fraction. Thus, in systems where the local population needs to persist during disturbance or dispersal events our findings suggest that a trait combination of an intermediate persistence ability and intermediate growth rate is optimal rather than maximizing either trait alone. Our results represent fundamental data on yeast dispersal and growth in single flowers which is necessary to simulate yeast dispersal and growth in larger landscapes.

This study revealed the importance of the remaining fraction in the nectar yeast system (i.e. the relative amount of the yeast population that remains in the flower after a pollination event) and more generally represents persistence ability against disturbance effects in other systems. For a better understanding of yeast population dynamics and dispersal it would be beneficial to test which properties of the flower, the pollinator and the yeast cells most affect the adhesion of yeast cells. Those might be shape, surface structure and hydrophobicity of the flower, the yeast cells and the pollinator mouthpart and the strength of movement (shaking) of the flower before and during the pollination event.

Mobile links shape metacommunities of nectar yeasts (Chapter 2)

After chapter one focused mainly on the disturbance effects of pollinators on the local nectar yeast population, chapter two describes the beneficial role of the pollinator as dispersal vector for microorganisms in nectar. By extensively sampling yeast metacommunities from nectar and pollinators, we report major changes in the composition of the yeast communities from pollinator to flower (Chapter 2). Through these efforts we identified how characteristics of the pollinator community affect various characteristics of yeast metacommunities. Our results show that the dispersal frequency of the mobile linker community was the main driver for species richness and abundance of the yeast metacommunities; however, species abundance was affected only by specific dispersal pathways (here: honeybees and bumblebees). The yeast species composition is primarily shaped by local processes like environmental filtering effects. Among the different pollinator groups, bumblebees and honeybees transported the most nectar-specialist species, wild bees and wasps the most insect-associated yeast species, and sawflies the most transient yeast species. These results could be explained by their specific nutrition, nesting substrates or life-history mechanisms. We found a

high overlap in yeast communities between flower and pollinator for social insects and low overlap for solitary insects. All yeast species in nectar except one were linked to pollinators and are consequently present in the pollinator metacommunity, whereas insect-pollinators (Brysch-Herzberg 2004; Herrera et al. 2010) transported more yeast species than were isolated from flowers. We recommend the nectar-yeast system with our new approach of identifying the insect-migrating dispersing species pool as a promising system for further studies on dispersal and colonization processes.

During the research on interactions of pollinators with nectar yeasts many open questions were addressed, but new questions were also raised. The effects of dispersal and disturbance on the nectar yeast community could not be disentangled because both occur simultaneously. In a full factorial laboratory approach both could be tested separately and combined to find out which process is more important for the resulting species composition. Our results indicate that bumblebees and honeybees are effective as yeast dispersers, whereas wild bees and flies showed the lowest potential to disperse nectar yeasts. This dispersal efficiency was indirectly inferred from combinations of data on community similarity and pollination frequencies but was not tested directly. Further experiments would be necessary to confirm our interpretation by measuring the dispersal efficiency of different pollinator groups on nectar-borne yeasts directly. In addition, to understand dispersal processes more exactly, it would be necessary to test if there is competition during zoochory. This might occur, for example, by occupation and blocking of dispersal opportunities or by creating substances that change the mechanical properties of the dispersal agent (e.g. substances with different hydrophobicity than the dispersing host organism).

Nectar yeast overwintering remains an open question (Chapter 3)

As shown in chapter two in late summer up to one third of all pollinators including bees, flies and wasps (own observations in 2016) and up to 71 % of bumblebees (Brysch-Herzberg 2004) transport nectar yeasts on the proboscis. Thus, it seems likely that nectar yeasts overwinter with bees or other pollinators (Brysch-Herzberg 2004). However, despite comprehensive sampling of three bee groups after hibernation described in chapter three, we could not find a single isolate of the nectar-specialized yeasts in early spring. Instead, we showed that some transient yeast species can use bees as hibernation sites. Some of those species, like *A. pullulans* and *D. hansenii*, are

found in nectar frequently and in high abundances, and some are only rarely reported in nectar, such as *F. wieringae*, *C. cygneicollum*, *C. magnoliae* and *Z. rouxii*. For these yeast species, bees are definitely a reliable hibernation pathway even if those yeasts can use other pathways as well. Contrary to the longstanding hypothesis that nectar-borne-yeasts hibernate with bees (Brysch-Herzberg 2004), we found no evidence to support this, and we instead found that bees support the hibernation of transient yeast species that compete with specialized nectar yeasts in flowers. This research indicates that bees are most likely not the primary habitat for overwintering in the nectar-yeast system.

To answer the question where they actually might be overwintering I suggest investigating ants, because of several reported yeast isolations from ants (Golubev and Bab´eva 1972; Middelhoven et al. 2003), their behavior to forage nectar and their ability to carry nectar yeasts (De Vega and Herrera 2012). Ants can even grow fungi for their own nutrition (Weber 2012) and store food in winter. Another possibility would be hibernation with solitary bees nesting in soil, which we did not test here. However, it could be possible that nectar yeasts do not rely on insects for overwintering at all. Hagen (1986) and Sporer (1996) report that the early research on nectar yeasts around 1950 was on their effect on cattle digestion. Cattle ingest nectar yeasts along with flowering plants in food and it was reported that nectar yeasts survive in the cattle rumen. Consequently, I suggest that vital yeasts are also part of cattle dung and may survive winter in this environment. In spring they could be taken up and transported to flowers by diptera that feed not only on rotten organic matter, but also occasionally from floral nectar. To understand the overwintering strategy of nectar yeast it might be helpful to compare population genetics of nectar yeast in late summer with those in early spring. A low genetic diversity in spring compared to late summer would indicate that overwintering is only rarely successful. A high genetic diversity already in spring would indicate that there are definitely undiscovered and effective pathways that ensure overwintering of a large part of the nectar yeast population.

Bottom-up effects of nectar yeasts on pollinators

In this thesis, research efforts focused on the effect of pollinators on nectar yeast; however, nectar yeasts can also have bottom-up backlash effects on pollinators and plant fertility. By changing nectar chemistry (Herrera, Garcia and Pérez 2008; Vannette, Gauthier and Fukami 2013; Good et al. 2014; Vannette and Fukami 2018), nectar scent

(Golonka, Johnson and Hinson 2014; Rering et al. 2017) and even flower temperature (Herrera and Pozo 2010) nectar yeasts can affect foraging behavior of pollinators. Increased attractiveness of nectar colonized with yeasts was shown by Herrera, Pozo and Medrano (2013) and Schaeffer et al. (2014). A higher nectar attractiveness should increase plant fitness, but only two studies with conflicting results are available on this topic: both a positive effect on pollen transfer rates (Schaeffer and Irwin 2014) and a negative effect on seed mass produced (Herrera, Pozo and Medrano 2013) have been reported.

Furthermore, previous studies on nectar yeasts and pollinator behavior tested only preferences of bees with unknown life history. Bees do not have an inborn preference for scents (Gould 1987). If we observe that a bee favors a specific scent, the only conclusion we can draw is that it must have previously learned to connect this scent with a reward. Thus, bees from different sites will show different preferences for different yeasts in flowers. To investigate which nectar yeast species has the highest impact on pollinator behavior, it would be necessary to test bees that are raised in a controlled environment and then test how quickly those bees can be conditioned with the different yeast scents or even the different volatile compartments of the yeast scent. Those studies could be conducted with proboscis extension reflex experiments (PER, Bitterman et al. 1983) and would show which scent can be best remembered by bees and therefore would be most likely favored under natural conditions. Nevertheless, previous studies showed that pollinators partly learned to favor nectar colonized by yeasts in natural environments. Contrary to the positive effects on nectar attractiveness, the nectar yeasts can have also detrimental effects on pollination. One major product of the yeast's fermentation process in sugar-rich nectar is ethanol. If the ethanol level reaches a certain level, pollinators may be repelled (Ehlers and Olesen 1997; Wiens et al. 2008).

Besides the reported studies that showed that nectar yeasts increase nectar attractiveness for pollinators, there are two studies by Vannette, Gauthier and Fukami (2013) and Good et al. (2014) that found no difference in pollinator preferences between nectar with and without yeasts. Those contradictory results show that we cannot generalize the first reported findings. From the perspective of the nectar yeast, a higher attractiveness to pollinators would increase dispersal events, but would this

really increase fitness over a longer time? Mittelbach, Yurkov and Begerow (2016) offer a broader view on this mechanism. They state that a preference of pollinators towards specific yeasts would even lead to reduced dispersal, because pollinators would forage mainly on flowers that are already colonized by nectar yeasts and thus reduce the chance for nectar yeasts to be transported to new uncolonized flowers. They concluded that conditions would be optimal for nectar yeast metapopulations if yeast do not change behavior of pollinators. This statement is in accordance with the results from chapter one showing that an increased pollination chance is detrimental to the size of a local yeast population and therefore reduces the overall number of dispersed cells. More precisely formulated, nectar yeasts would gain the highest benefit from passive dispersal if they avoid detection by pollinators (Mittelbach, Yurkov and Begerow 2016).

Nevertheless, a preference of pollinators for yeasts has been reported by some studies and accordingly one must ask: Why should bees favor nectar colonized by yeast? The altered nectar scent with the additional volatiles from nectar yeast metabolism could be an signal that helps bees to recognize the availability of nectar more easily than the scent of nectar alone (Pozo et al. 2009). Additionally, yeasts could serve as an additional nitrogen source besides pollen, which is the only nitrogen source for bees, as far as we know, or the yeasts may even help with the bee's digestion in the gut. Despite these potential mechanisms a fitness effect of nectar yeasts on pollinators has not been found to date (Schaeffer et al. 2017).

Conclusion and outlook

The discussion of bottom-up effects of nectar-borne yeasts shows that there is a dearth of evidence and contradictory results on how strong yeasts can affect their host-plant or their pollinator community. The top-down effects that pollinators have on the ecology of nectar yeasts can be very strong. From the results of this thesis I conclude that the local populations up to metacommunities of nectar yeast are mainly controlled by the characteristics of the pollinator assemblage, primarily by pollinator species richness, composition, abundance and foraging decisions.

Nectar yeasts are not classical parasites, though they could potentially ferment nectar to a totally pollinator-unattractive medium by production of ethanol. They can reduce seed production of their host plant slightly (Herrera, Pozo and Medrano 2013), but have

positive effects on plant fitness, as they may increase flower attractiveness (Herrera, Pozo and Medrano 2013; Schaeffer et al. 2014). They are also not classical symbionts, since plants and pollinators do not depend on them for any important process. As we do not know more about their function in the ecosystem, nectar yeasts seem to be coexisting in the nectar, profiting from the plant-pollinator system while affecting it just slightly. Perhaps it is exactly that strategy that makes them successful in the nectar environment and ensures survival.

This thesis illustrates that the life of nectar yeasts depends strongly on pollinators and that the strategy of passive dispersal can have extensive consequences on the environmental conditions, metacommunity characteristics and lifecycle for a microbial system. Thus, the value of pollinators is not only their pollination service, but also their dispersal service to microorganisms, by which they give life to and drive the nectar yeast system. Consequently, the recently observed global decline of pollinators (Potts et al. 2010) threatens the survival of nectar yeast populations too. This research contributes to our basic knowledge on nectar yeast ecology, especially on their dependence on flower-visiting insects and encourages other scientists to apply nectar yeasts as a microbial model system for general ecological questions. May this work support the conservation of nectar yeasts as a fascinating part of the microbial diversity of our environment.

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DECLARATION OF AUTHORSHIP

I hereby declare that the thesis submitted is my own unaided work. All direct or indirect sources used are given as references. All contributions of co-authors are acknowledged.

Berlin, October 11th 2018 Sebastian L. Hausmann

APPENDICES

APPENDIX 1: SUPPLEMENTARY MATERIAL CHAPTER 1

Content

A: Model description

To understand the effect of many consecutive pollination events on population size and dispersal potential we developed a stochastic simulation model (NetLogo 5.3.1; Wilensky 1999) of nectar yeasts in one single flower. The model calculates the population size and the amount of dispersed cells of a single nectar yeast population over time, dependent on: pollination time and chance, inoculated cells during first pollination event, transmitted cells to the next flower, cells that remain in the flower during pollination, nectar production rate and growth rate of yeast cells with lag phase.

Modelling was done with NetLogo 5.3.1 (Wilensky 1999). The model works stochastically, exclusively with global variables without individuals or space. One time step is one hour.

A1: Model functions

Scheduling:

- observer sets all input variables
- nectar production
- growth
- pollination

- lagphase
- counting and controlling
- stop
- output

The observer sets the following input variables:

- number of added cells at first pollination event (startcells, 0 to 1000)
- number of added cells for later pollination events (addedcells, 0 to 1000)
- chance that pollinator carries yeast cells (beeswithyeast_chance: 0 to 100%
- growth rate of yeast cells per hour in percent (growthrate, 1 to 25 %)
- remaining fraction of cells during pollination event in percent (remainingcells, 1 to 100%)
- lag phase in hours, time between cell inoculation and cell growth starts (lagphase, 1 to 24 hours)
- maximum number of yeast cells if maximum nectar is available (maxcells, 1 to 100.000)
- chance of pollination event per hour (pollination_chance, 1 to 100%)
- maximum number of pollination events until flower is fully pollinated (maxpollinations, 1 to 10)
- time when pollinators are active (polltimemin and polltimemax, 1 to 24 hours)
- maximum number of days the model is running (maxdays, 1 to 10)
- nectarproduction per hour (nectarprodperh, 10 to 100%)
- maximum amount of nectar in µl (maxnectar, fixed to 1 in our simulation)
- probability that dispersal to the next flower is successful (dispersalchance: 0 to 100%)
- percent of cells that will be dispersed if dispersal is succesful (dispersalrate, 0.1 to 10 %)

Nectar production: Nectar production per hour that takes place if the flower is not fully pollinated and the maximum amount of stored nectar is not reached. The nectar production per hour is set by the observer in the beginning. From the current amount of nectar, the current maximum possible number of yeast cells is calculated. If nectar reaches a maximum amount the number of maximum cells that can live off that resource is the maximum cell number that was set from the observer in the beginning. If the

nectar is less than the maximum the same factor reduces the number of possible yeast cells.

Growth: Occurs only if nectar is not empty and only for those cells that are not in the lag phase. Growth function is logistic and limited by the maximum possible number of yeast cells (depending on nectar amount). The growth rate in the equation is set by the observer in the beginning.

New cell number=old cell number + (growth rate * old cell number *(1-old cell number/max possible cells))

Pollination: Can happen by chance if flower is not fully pollinated yet and pollinators are active. The number of old cells and cells in lag phase is reduced to the percentage of remaining cells. Cell number cannot be reduced below 1 and population of old cells cannot be reduced below the population number after the last pollination event. That is, because we assume it is more likely for a cell that remained one time in the flower to also remain in the flower during consecutive pollinations events. The other part of cells (that does not remain) becomes the uptaken cells by the pollinator. Nectar is reduced to zero and number of pollinations is counted plus one. After reducing the current cell population new cells are added to the total number of cells, but new cells start as part of the lag phase group. If pollination occurs, the chance that yeast cell inoculation happens is set by the variable beeswithyeast_chance. If inoculation is successful the number of added cells is defined by the variables startcells (if it is the first pollination) or addedcells (if it is not the first pollination).

In the last step of the function the number of cells that emigrate with the pollinator to the next flower will be calculated. If this emigration is successful depends again on the variable dispersalchance. If emigration is successful dispersedcells is set to dispersalfactor times the number of uptaken cells by the bee.

Lag phase: Every newly introduced cell has its own counter for the lag phase that increases by 1 for every time step. If the duration of the lag phase has passed the cell is added to the number of old cells that can grow in the growth function. The function is programmed in NetLogo with an array.

Counting and controlling: Number of hours is increased by 1 every time until 24, then it starts with 0 again. After 24 hours day count is increased by 1.

Stop: If number of days is equal to maximum days, the model stops, otherwise it starts from the beginning. If number of pollinations is equal to maxpollinations the model stops, too.

Output: Output variables are the total number of dispersed cells and the highest population number reached within the simulation.

Random variables are pollination chance per hour, dispersalchance per pollination event and the chance that bees transport yeasts (beeswithyeast_chance). Pollination chance can be set from 0 to 100% and is limited by several environmental aspects. It can only happen during the pollinator-active period (polltimemin till polltimemax), if nectar is not zero (not attractive) and if flower is not fully pollinated. 25 % chance means that on average every four hours a pollination event can occur, 50 % chance means every two hours and so on. Dispersalchance can be set from 0 to 100% and regulates by chance if the dispersal of yeast cells into the flower (inoculation) or the dispersal to the next flower (emigration) is successful or not. Beeswithyeast_chance can be set from 0 to 100% and is a requirement that yeast cells can be inoculated during pollination.

A2: Input variables

number of start cells / number of added cells

The “number of startcells” is the number of yeast cells a bee is introducing to a flower during the first pollination event. The “number of added cells” means the number of cells that is introduced during every following pollination event. Both values can be set from 1 to 1000. To use two variables for the same value makes it possible to set yeast cell inoculation only for the first pollination event. We did not find any studies that give values for these variables. The values we used come from calculations of original yeast densities (see input description of maximum cell density) combined with results from our lab experiments (see method part of manuscript).

number of highest possible cell densities

The highest possible cell density limits the growth of the yeast population when resources start to be in short supply. Reported cell densities of natural systems show values between 7x103 cells µl-1 (De Vega and Herrera 2012) to 3.64x106 cells µl-1 (de Vega, Herrera and Johnson 2009). Carlos M. Herrera, De Vega, Canto, & Pozo (2009)

investigated 22 different herbaceous plants in Spain and Mexico and concluded that cell densities under natural conditions are likely to be below 4x105 cells µl-1 in floral nectar. Therefore, our default maximum cell density is set to 4x105 cells µl-1 cells.

Appendix Table 1: Nectar yeast cell densities in average and reported maximum from literature review

Reference	Plant species	Highest reported cell density in cells μl^{-1} (±SE)
De Vega and Herrera 2012	*Cytinus hypocistis* *Cistus ladanifer* *Cistus salviifolious* *Halimium halimifolium*	peak 62 000, mean 4100 ±590 mean 3600 ± 1200 mean 6000 ± 1300 mean 6000 ± 2100
Pozo, Herrera and Bazaga 2011	*Aquilegia cazorlensis (out of 12 herbacious plant species)*	*mean* 11 127
Brysch-Herzberg 2004	*Digitalis purpurea*	highest value 16 000
Herrera, Garcia and Pérez 2008	*Aquilegia vulgaris* *Aquilegia. p. cazorlensis* *Helleborus foetidus*	highest value 43 003, mean 11 362 ± 3603 highest value 156 800, mean 37 166 ± 24 284 highest value 219 545, mean 36 612 ± 14 956
Herrera *et al.* 2010	*Helleborus foetidus*	highest value 127 622, mean 17 821 ± 2844
Herrera and Pozo 2010	*Helleborus foetidus*	highest value 194 000, mean 48 200 ± 7 900
Herrera *et al.* 2009	*22 different herbaceous plant species*	highest value 412 036
de Vega, Herrera and Johnson 2009	*Moraea graminicola*	highest value 3 640 333, mean 881 062

Growth rate

This value (growthrate) gives the proportional growth per hour of the nectar yeast population. It can be set between 0.1 to 25.0 %. 12 % per hour equals 15 times increase per day (growth rate ^24). The value we use here is the maximum growth rate during the exponential growth phase of the yeast. In experiment A we could also measure growth rates. The exponential growth phase was identified between the second and the third day (24 to 48 hours). The relative growth rate was calculated as the optical density (OD) value at 48 hours divided by the OD value at 24 hours.

The main resource for nectar yeast growth is nitrogen. Highest growth rates were found by Mittelbach, Yurkov, Stoll, & Begerow (2015) when media were enriched with 0.5 mM casein digested amino acids. The higher the sugar concentration in nectar the higher the osmotic pressure resulting in smaller growth rates for some species. Very slow growth rates were found by (Mittelbach et al. 2015) when they tested growth of 4 yeast species

under an extreme high sugar concentration of 40%. Data from both last cited studies are estimated from plots and lack standard errors.

Appendix Table 2: Nectar yeast growth rates from literature review

Reference	**Medium**	***Species***	**Average Increase per day (±SE)**
Experiment A	25% sugar Nitrogen source: casein hydrosilate 0,32 mM	*Aureobasidium pullulans* *Candida rancensis* *Cryptococcus victoriae* *Metschnikowia reukaufii*	13.9±1.6 13.2± 0.8 7.2±0.5 10.2±1.1
Mittelbach *et al.* 2016)	30% sugar nitrogen source: casein digested amino acids 5 mM).	*Metschnikowia reukauffi* *Candida rancensis* *Cryptococcus victoriae* *Udeomyces pannonicus*	~100 ~100 ~4 ~65
Mittelbach *et al.* 2015	40% sugar Unknown nitrogen source	*Cryptococcus canescens* *Cystofilobasidium capitalum* *Metschnikowia reukauffi*i *Candida rancensis*	1.1 1.2 1.4 1.6

As the model uses hours we calculated the growth rate per day with the 24th root of the daily growth rate and subtracted 1 to obtain the relative increase per hour: (X^(1/24))-1. In the model you can set the growth rate per hour from 0.5%, which means 1.3 times increase per day to 25.0%, which means 211.7 times increase per day. Highest realistic value is 21%, which equals 97 times increase per day.

maxpollinations and pollination chance

The number of flower visits a flower needs to be fully pollinated and stops nectar production is very different among plant species and depends mainly on the kind and behavior of the pollinators. For the number of pollination events needed there are too few reports in the literature to use as reference. Many studies do not give the number of flower visits but the total time all pollinators spend pollinating until the flower closes. Unfortunately, this cannot be used for the model. Nevertheless, for some plant species there are observations that help estimate the pollinator frequency per single inflorescence. Pollination chance can be expressed as the time between two pollination events and can be quite different depening on plant species and ecosystem and varies between 1.49 to 21.7 hours. Pollination chance can be set from 0 to 100%. 1% chance means on average a pollination event every 100 hours, 10% means every 10 hours, 20% means every 5 hours and so on. Before the simulation starts the model gives back the

mean hours between 2 flower visits depending on the set pollinaton chance. As we did not find reliable data for maxpollinations we set this variable to 10.

Appendix Table 3: pollinator frequencies from literature review

Reference	**Plant species**	**pollinators**	**Mean Hours between 2 visits per single flower**
Nedić *et al.* 2013	*Brassica napus* in Serbia	honeybees	1.49 h
Eckhart *et al.* 2006	*Clarkia xantiana* in USA	All pollinators	2.11 h
Hennig and Ghazoul 2011	4 herbaceous plants in Zürich, Switzerland	All pollinators	3.24 h
Hausmann, Petermann and Rolff 2016	4 tree species in Berlin, Germany	All pollinators	4.2 h
Martinez and Armesto 2005	26 tropical plants in Chile	All pollinators	24.7 h

Nectar production per hour, maximum amount of nectar, open flower time

Realistic values for nectar production per hour were taken from several studies. To keep the model simple, the maximum amount of nectar should be kept to 1(µl). It calculates yeast density as the number of cells per µl, which is commonly used to compare yeast densities. If the plant species that will be modelled does produce more than 1 µl as the highest nectar volume the results of the model can be multiplied with the realistic volume. Default value for maximum nectar amount is 1, for nectar production per hour it is 10% and for open flower time it is 5 days.

Appendix Table 4: highest nectar amount, nectar production per hour and number of days a flower is open from literature review

Reference	**Plant species**	**highest nectar**	**Nectarpro-**	**Open for**

		amount	duction	x days
Nedić *et al.* 2013	*Brassica napus*	5.93 µl	0.245 µl/h	3
Pierre *et al.* 1999	*Brassica napus*	2.33 µl	-	-
Antoń and Denisow 2014	*Aconitum lycoctonum* *Aconitum carmichaelii*	8.60 µl 21.70 µl	0.112 µl/h 0.095 µl/h	3.2 9.5
Pleasants 1983	*Ipomopsis aggregata*	4.77 µl	0.252 µl/h	-
Farkas *et al.* 2012	*Allium ursinum*	1.66 µl	0.069 µl/h	-
Leshem, Keasar and Shmida 2011	*Salvia hierosolymitana male* *Salvia hierosolymitana female*	-	0,290 µl/h 0,790 µl/h	2.0
Lu *et al.* 2015	*Aconitum gymnandrum*	male phase: 0.72 µl female phase: 0.63 µl	-	6 to 10
Pengelly and Cartar 2011	*Chamerion angustifolium* *Delphinium glaucum* *Mertensia paniculata* *Vicia americana*	0.45 µl 0.62 µl 1.75 µl 1.01 µl	0,019 µl/h 0,026 µl/h 0,073 µl/h 0,042 µl/h	-
Jennersten and Nilsson 1993	*Viscaria vulgaris*	-	-	4

polltimemin and polltimemax

In the model it is possible to change the time at which pollinators are active. We set it by default from 8 to 18 o´clock according to personal observations during insect sampling for other research projects in summertime in central Europe.

beeswithyeast_chance

Mittelbach, Yurkov, Stoll, & Begerow, 2015 found in 42 % virgin flowers nectar yeasts after a single flower visit by honeybees. We found 61.1% inoculation chance for the first visited flower after nectar uptake when bees transported yeasts in Experiment C. If we combine both values we can calculate that around 70% of bees observed by Mittelbach et al., 2015 transported yeast cells. Therefore, we will set the default value for beeswithyeast_chance to 70% in the model to get a representative inoculation rate.

remaining cells

This value is between 1 and 100% for the number of cells that remain in the flower after nectar uptake by a pollinator. It was investigated in experiment A.

dispersalrate (dispersed cells)

Gives the probability between 0.1 and 1% of cells that are transported via the bee to another flower from the total number of cells the bee takes up from the current flower together with the nectar. This was investigated in experiment C.

dispersalchance (dispersal probability)

Dispersal chance can be set from 0 to 100% and regulates by chance if the dispersal of yeast cells into the flower (inoculation) or the dispersal towards the next flower (emigration) is successful or not. It was studied in experiment C.

lag phase

Lag phase means the time a yeast cell needs to adjust to a new environment until it starts growing. It can be set from 1 to 24 hours.

A3: Sensitivity analysis

In the following sensitivity analysis, the effect of different variables on the response variable highest cell number at the end of the simulation was tested (see Table 5). The difference between smallest (min) and highest (max) value of the tested variable can be at the most 4x105 cells µl-1 yeast cells per µl as this is the highest possible number of cells. The cell number difference is the mean of the highest value minus the mean of the smallest value of the highest cell number per simulation. A positive difference of cell numbers means the higher the value of the variable the larger the population. A negative difference of cell numbers means the higher the value the smaller the population. The default values of the model that were not changed in general are shown in column "default". Every setting was tested 100 times. Maximum amount of nectar is set to 1 by default and will not be tested here, because its effect depends always on the nectar production per hour, which is already tested. Dispersal rate will not be tested because it only has an effect on the response variable "total dispersed cells" which is not yet important for understanding the model parameters.

Appendix Table 5: Default, minimum and maximum values, and results from sensitivity analysis

variable	default	test min	test max	cell number difference
growthrate	10%	1%	25%	51 918
pollltime	8 to18	8 to 9	8 to 24	-23 690
pollchance	33%	1%	100%	-20 522
remainingcells	30%	1%	100%	12 289
addedcells	10	1	200	4 461
startcells	10	1	200	3 832
maxpollinations	10	1	100	598
maxdays	5	1	10	544
dispersalchance	60%	1%	100%	457
beeswithyeast	70%	1%	100%	432
lagphaseh	10	1	24	-237
nectarprod	10%	10%	100%	131
maxcells	400 000	400 000	1 000 000	86

The sensitivity analysis showed the highest effect size for growthrate (51 918). A high effect size was found for pollination chance (-20522), too. The smaller the pollination chance the higher the yeast population. A small population chance can lead to no pollination at all but if a pollination occurs the yeast cells can grow to high numbers without being removed from the flower again. Also, a very high effect size was found for polltime (-23 690) which can be explained by the same reason as for pollination chance. The shorter the time period during which pollination can occur the smaller the number of pollination events. Also important is the effect size of remaining cells (12 289). The number of starting cells and added cells have effect sizes of 3 832 and 4 461. The amount of introduced cells is not so important for this model since after first inoculation exponential growth leads already to huge cell numbers. If one cell was already inoculated additional inoculations are not needed to reach high population densities. For other systems without exponential growth this may be more important. Very small effect sizes were found for maxpollinations, maxdays, dispersalchance, beeswithyeast, lagphaseh, nectarprod and maxcells. Some of them may have bigger effects with different settings. For example, maxcells can only show its large effect size when cells have a large growth rate. In comparison, with this setting growth rate is around 2 times more important than pollinationchance and five times more important than remaining cells. In summary, growthrate, followed by the dynamics of pollination events (pollchance and polltime) and remaining cells have the highest influence on the results. Effects of polltime will not be tested in the main analysis because it is highly correlated with pollchance, which will be tested.

A4: Description of main analysis and default values

From the sensitivity analysis of the model we knew that growth rate, pollination chance and remaining fraction have the greatest impact on yeast population size. We therefore systematically analyzed the effects of these three variables plus an additional variable for inoculated cell numbers on yeast performance in our simulations.

We performed model experiments to determine the impact of four uncertain but sensitive parameters (initial inoculated cell number, growth rate, pollinator frequency and remaining fraction) on the performance of the yeast population. Each of the three sensitive parameters was separately varied, while keeping the other two sensitive parameters fixed at their default value (see below). This was done for three levels of inoculated cells.

Changes in growth rates were tested from 5 to 23% in steps of 1%; for pollination chance from 0 to 20% in steps of 2%, from 20 to 40% in steps of 5% and from 40 to 100% in steps of 10%; for proportions of remaining fraction from 0 to 100% in steps of 10. We did 1000 repetitions per setting. Default values were 10% growth per hour, 33% pollination chance per hour and 30% remaining fraction. One additional analysis was run testing change in growth rates with optimized fixed value for remaining fraction of 50% which is close to the highest value found in Experiment A for CR (not shaken). Highest population size and total number of dispersed cells (to the next first visited flower) were measured per repetition.

Inoculated cell numbers and dispersal probability

Inoculated cell numbers were calculated based on results from experiment C and realistic source populations of 103 (low), 104 (intermediate) and 105 (high) cells µl-1. If dispersal is successful average percentage of inoculated cells in Experiment C at the first flower over all cell densities for MR was 0.282% (±0.060% SE) and 0.245% (±0.048%) for CR. We set it to 0.25 %. The percent of dispersed cells from the total cells of the source population is calculated as "(1 - % persistence ability) * % dispersed cells", for our example "(1-0.3) * 0.0025 = 0.00175". Therefore, 0.175% of the source population would be dispersed and start our simulated population. For our three inoculation scenarios that means our modeled flower will be inoculated with 2 (low source population: 103 cells µl-1), 18 (intermediate source population: 104 cells µl-1), and 175

(high source population: 105 cells µl-1) cells during pollinations, if dispersal is successful. We found 61.1% inoculation chance when we knew that bees transported yeasts in Experiment C. In the model, it will be set to 60% as well for inoculation and dispersal.

Growth rate

Observed growth rates of nectar yeasts in lab experiments result in a range between 1.1 to 100.0 times population increase per day (see supplementary material). We will set a default growth rate of 10% per hour, which is equal to 9.85 population increase per day, and this is near the observed growth rates in our experiment A and a tenth of the highest and ten times higher than the lowest observed rates from other studies.

Pollination chance

In European ecosystems pollination occurs every 1.5 h (Nedić et al. 2013) to 4.2 h (Hausmann, Petermann and Rolff 2016). Default value for pollination chance in our simulations was set to 3.0 h (33% pollination chance) to simulate an intermediate and realistic pollinator density from Europe.

Remaining fraction

Remaining fraction is given in percent of the current yeast population and set by default to 30%, which is close to the mean remaining fraction for CR in Experiment A (33.1% ±14.5). Other variables were set as in the sensitivity analysis (supplementary material B3). One additional analysis was run testing change in growth rates with optimized fixed value for remaining fraction of 50% which is close to the highest value found in Experiment A for CR.

B: Detailed materials and methods of lab experiments

B1: Experiment A, remaining fraction

In experiment A we measured the remaining fraction, which is the proportion of yeast cells that remain in a flower when nectar is removed. To mimic this process experimentally, we used the wells of two 96-well plates (greiner bio one, cat# 655180) to represent flower cups. On each plate we cultivated the two yeast species with four different nectar exchange treatments plus control without yeasts. Each treatment was

replicated in 8 wells. Fungi were grown on a 1% sugar agar plate (0.3 % yeast-extract, 0.3 % malt-extract, 0.5 % peptone, 1.0 % dextrose, 2.0 % agar, 95.9 % water, values in % w/w) to verify purity and viability of the strains. During the experiment, yeasts were grown in 100µl artificial nectar (25% sucrose w/v solution supplemented with 0.32mM amino acids from casein hydroxilate) starting with a density of 20 cells µl-1. Cell numbers were counted with a Neubauer Counting Chamber. Artificial nectar was filter-sterilized with Rotilabo syringe filters 0.22µm (Carl Roth GmbH and Co. KG, article# KH54.1) before use. The samples were incubated at 25°C. Nectar was exchanged never, once (after 48 hours), twice (additionally after 72 hours), or three times (additionally after 96 hours), representing pollination events. For nectar exchange the entire artificial nectar was removed with pipettes and fresh artificial nectar was added. To test for effects of agitating (mimicking the physical effect of a pollinator) one of the two 96-well plates was shaken for ten seconds before nectar removal. Before and after each nectar exchange optical density was measured at 660nm with a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories GmbH, Munich, Germany).

B2: Experiment B, transported cells

In experiment B we measured how many yeast cells stick to the proboscis of a bee when drinking nectar. Cells on the proboscis are transported cells that can be potentially dispersed. The difference to experiment A was that a real bee removes the sugar water from the cup (cap of a PCR Eppendorf tube) and sugar water (25% w/v sucrose solution) was sucked within 1 to 5 mins after it was placed into the cup (contrary to every 24 hours in experiment 1). We could not observe a settling of the yeasts on the bottom of the well within that time. So, we expect the bees to consume almost all cells with the sugar water. We measured the number of transported cells after drinking 10 µl nectar containing a defined density of cells of one of the two yeast species (102, 103 and 104 cells µl-1). Cell numbers were counted with a Neubauer Counting Chamber. Yeast species were prepared as in experiment A, but 25% w/v sucrose solution without nitrogen source was used to suppress growth of yeast cells to be sure that cell numbers counted in the morning are equivalent to cell numbers in the evening when cups were streaked on agar. Bees were kept in sterile glasses at the hive-entrance when bees were leaving the beehive and fed with the yeast sugar water in a small cup. The hives were placed in a greenhouse-flight room at Freie Universität. The experiment was conducted

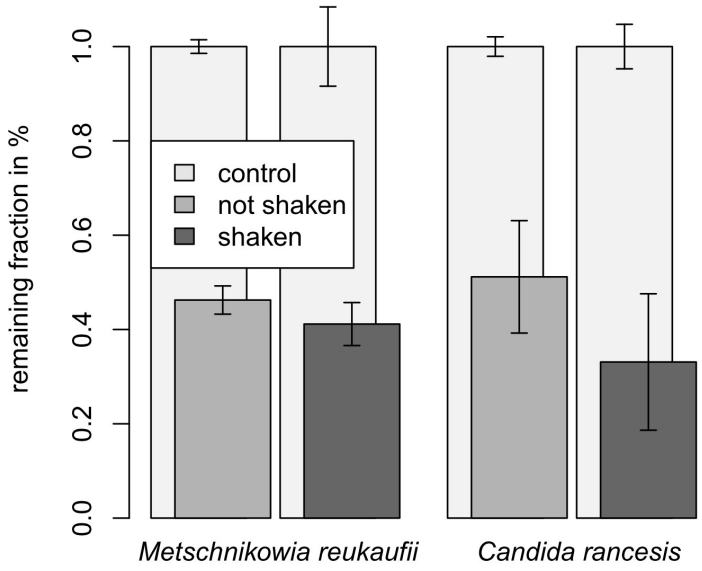
in December 2016. Kept bees were fed with 10 µl yeast media. For every treatment five bees were used. After the bee emptied the cup it was killed with CO2 gas. Probosci of the bees were removed with forceps and vortexed with 50 µl sterile water to separate yeast cells from the proboscis. Every proboscis together with the water was spread on 1% sugar agar plates. Agar plates with samples were incubated at 25°C. After five days the number of colony forming units (CFUs) was counted. The proportion of transported cells on the proboscis from uptaken cells was calculated. Five bees fed with pure sugar water (control) instead of sugar water with yeasts (treatments) had on average 1.2 (±0.7 SE) CFUs on the proboscis. This control value was subtracted from absolute CFU measurements of treated bees.

B3: Experiment C, dispersal probability and number of dispersed cells

Experiment C was designed to measure how many yeast cells are dispersed by a pollinator from one source flower to the first and the second consecutive visited flower. We used honeybees and a plexiglass tunnel with five consecutive chambers that could be handled from outside the tunnel. Yeasts were prepared as in experiment B, and bees were kept as described in experiment B. Bees were placed into the tunnel and passed the chambers. Doors to the next chamber were opened when bees emptied the cups (see below). In chamber two to five we placed the cap of a PCR Eppendorf tube with 10 µl of 25% w/v sucrose solution. In the third chamber, we added one of the two yeast species with the same cell densities as used in experiment B. Cell numbers were counted with a Neubauer Counting Chamber. The caps in chamber four and five will be called cup 1 and cup 2 and represent artificial flowers. For every yeast species and cell density 15 different bees were used. After the bee drank the medium the cups were flushed with 50 µl sterile water in a closed PCR Eppendorf tube, streaked on agar plates, incubated at 25°C for 5 days and CFUs of yeasts were counted. In chamber two we measured how many yeast cells the bee already carried before the experiment (control), and in chambers four and five how many yeast cells the bee dispersed after experimental yeast uptake from chamber three to the first (cup1) and second (cup2) visited cup. The proportion of dispersed cells from uptaken cells was calculated after subtraction of CFUs from the control plate.

C: Plots and tables for results of experiments

C1: Experiment A, remaining fraction



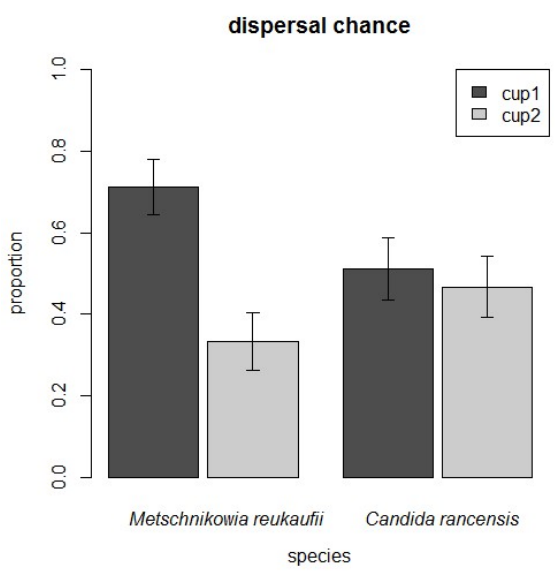
Appendix Figure 1: Proportion of remaining fraction after nectar exchange for *Metschnikowia reukaufii* and *Candida rancesis* for treatments that were shaken and not shaken before nectar exchange. Control is the reference value from treatments without nectar exchange. Error bars represent standard error (n = 8).

C2: Experiment B, transported cells

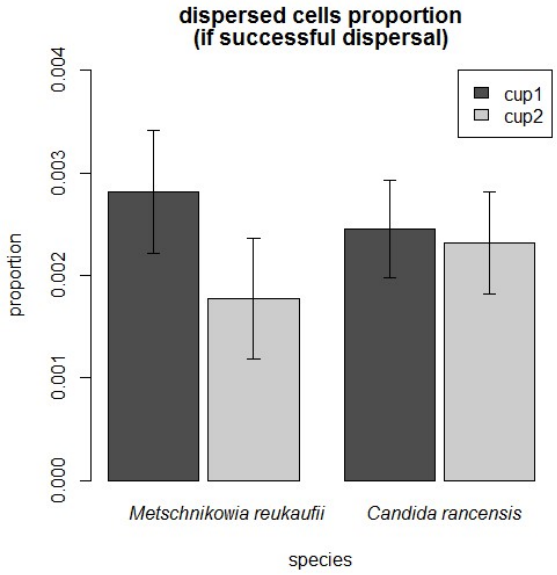
Appendix Table 6: Cells that were transported on the bees' proboscis as absolute number and proportion of the uptaken cells. Results are given for two species, *Metschnikowia reukaufii* and *Candida rancensis* and 3 cell densities plus control without yeast cells.

Cell density/µl	***M. reukaufii*** (± SE)		***C. rancensis*** (± SE)	
	CFU counts	**CFU percent**	**CFU counts**	**CFU percent**
0	1.2 (± 0.7)	-	1.2 (± 0.7)	-
100	52.8 (± 16.5)	5.28 % (± 1.65)	2.6 (± 0.6)	0.26 % (± 0.06)
1 000	892.5 (± 179.6)	8.93 % (± 1.80)	344.7 (± 77.5)	3.45 % (± 0.77)
10 000	1846.2 (± 257.4)	1.85 % (± 0.25)	9893.3 (± 1942.0)	9.89 % (± 1.94)

C3: Experiment C, dispersal probability and number of dispersed cells



Appendix Figure 2: Dispersal chance of nectar yeasts from source cup to two following cups by a honeybee. Differentiated for two nectar yeast species and the two consecutively visited cups. Error bars represent standard error (n=45).



Appendix Figure 3: Proportion of dispersed cells as a fraction of total cell number taken up by the bee from source cup to two following cups by a honeybee. Differentiated for two nectar yeast species and the two consecutively visited cups. Error bars represent standard error (n=45).

Appendix Table 7: Dispersal chance, proportion of dispersed cells if dispersal was successful and resulting value for proportion of dispersed cells (product of both values) for two yeast species, two consecutive visited cups and three yeast cell densities. M.: *Metschnikowia reukaufii*; C.: *Candida rancensis*

Cell Density (cells/µl)	**species**	**cup 1** (± SE)			**cup 2** (± SE)		
		dispersal chance	**dispersed cells proportion (if successful)**	**dispersed cells proportion**	**dispersal chance**	**dispersed cells proportion (if successful)**	**dispersed cells proportion**
100	***M.***	60.0% (±13.1)	0.356% (±0.053)	0.213% (±0.056)	13.3% (±9.1)	0.400% (±0.100)	0.053% (±0.038)
1.000	***M.***	80.0% (±10.7)	0.208% (±0.104)	0.167% (±0.086)	66.7% (±12.6)	0.159% (±0.077)	0.106% (±0.054)
10.000	***M.***	73.3% (±11.8)	0.301% (±0.128)	0.221% (±0.099)	20.0% (±10.7)	0.090% (±0.078)	0.018% (±0.016)
100	***C.***	45.7% (± 13.3)	0.329% (±0.087)	0.153% (±0.058)	40.0% (±13.1)	0.283% (±0.070)	0.113 % (±0.046)
1.000	***C.***	53.3% (±13.3)	0.196% (±0.104)	0.105% (±0.060)	40.0% (±13.1)	0.038% (±0.010)	0.015% (±0.006)
10.000	***C.***	53.3% (±13.3)	0.222% (±0.053)	0.118% (±0.040)	60.0% (±13.1)	0.326% (±0.088)	0.196% (±0.067)

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APPENDIX 2: SUPPLEMENTARY MATERIAL CHAPTER 2

A: Additional tables and figures

Tables

Appendix Table 8: Classification of isolated yeast species to ecological groups depending on their main habitat or the main source of isolations based on species descriptions by (Kurtzman, Fell & Boekhout, 2011).

yeast species	ecological group
Aureobasidium pullulans	transient species
Debaryomyces hansenii	transient species
Debaryomyces marama	transient species
Dioszegia hungarica	transient species
Dothiora cannabinae	transient species
Filobasidium chernovii	transient species
Filobasidium oeirensis	transient species
Filobasidium wieringae	transient species
Holtermaniella takashimae	transient species
Leucosporidium scotti	transient species
Papiliotrema laurentii	transient species
Sarocladium strictum	transient species
Sporobolomyces metaroseus	transient species
Vishniacozyma victoriae	transient species
Candida bombi	insect-associated
Metschnikowia kunwiensis	insect-associated
Metschnikowia pulcherrima clade	insect-associated
Starmerella bombicola	insect-associated
Candida rancensis	nectar-specialist
Metschnikowia gruessii	nectar-specialist
Metschnikowia reukaufii	nectar-specialist

Appendix Table 9: Overview of pollinator visitation frequency observed from three different tree species and the proportions of different insect groups of flower visits (Fig 10a). Means of flower visit proportions and Shannon diversity are calculated from five values representing five different trees. *flower visits means per 1000 flowers within 30 minutes. **Shannon diversity from flower visit proportions of different insect groups.

	Acer	*Robinia*	*Tilia*	Overall
Total number of flower visits	2 848	3 047	11 719	17 614
Pollinator visitation frequency*	474.7 (±34.3 SE)	222.8 (±65.5)	781.3 (±143.1)	-
Shannon diversity**	1.1 (±0.1)	0.6 (±0.1)	1.29 (±0.05)	-
Flover visits honeybees (%)	3.6 (±1.8)	15.9 (±4.8)	33.6 (±3.7)	21.4 (±3.1)
Flover visits bumblebees (%)	17.6 (±3.9)	75.3 (±5.7)	40.3 (±3.9)	50.4 (±4.7
Flover visits wild bees (%)	53.8 (±10.7)	5.8 (±2.9)	4.6 (±1.0)	13.5 (±3.8)
Flover visits wasps (%)	2.3 (±1.2)	0.4 (±0.4)	10.0 (±2.4)	4.8 (±1.3)
Flover visits sawflies (%)	0.5 (±0.5)	0.5 (±0.5)	5.1 (±1.2)	2.5 (±0.68)
Flover visits other Flies (%)	18.0 (±6.7)	0.4 (±0.4)	4.8 (±1.0)	5.3 (±1.6)
Flover visits beetles (%)	3.3 (±3.0)	1.2 (±0.6)	0.2 (±0.1)	1.1 (±0.6)
Flover visits butterflies (%)	0	0.5 (±0.4)	1.5 (±1.2)	0.8 (±0.5)
Flover visits other insects (%)	0.8 (±0.8)	0	0	0.1 (±0.1)

Appendix Table 10: Results of the linear mixed models for yeast species richness (sp. richn.) and proportions of samples with yeasts (samples w. y.) from nectar and pollinators (poll.) using tree ID (n = 15) as a random effect (Appendix Fig. 9). Plant distinguishes between the three sampled tree species. Subsets of honeybees (honeyb.) and bumblebees (bumbleb.) represent only the yeast species pool that were transported by the specific pollinator group. num d.f.: numerator degrees of freedom; den d.f: denominator degrees of freedom.

	Dependent variable	Independent variables	Num d.f.	Den d.f.	F value	p value
a)	sp. richn. nectar	sp. richn. poll	1	17	13.1	0.002
		plant	2	12	4.3	0.038
		sp. richn. poll * plant	2	17	0.2	0.830
	sp. richn. nectar (only bumbleb.)	sp. richn. poll.	1	17	14.1	0.002
		plant	2	12	1.7	0.226
		sp. richn. poll. * plant	2	17	0.2	0.802
	sp. richn. nectar (only honeyb.)	sp. richn. poll.	1	17	16.7	<0.001
		plant	2	12	7.2	0.008
		sp. richn. poll. * plant	2	17	0.2	0.856
b)	nectar-samples w. y.	poll.-samples w. y.	1	17	7.7	0.013
		plant	2	12	1.9	0.194
		poll.-samples w. y. * plant	2	17	0.5	0.618

Appendix Table 11: Results of t-tests between observed bipartite network indices (Dormann et al., 2008) of the pollinator-yeast-network (Fig. 10b) and the average of ten null-models. "obs" is the observed value from the original network, "null mean" is the average value from the ten null-models, "lower CI" and "upper CI" give the lower and upper limit of the 95% confidence interval.

pollinator-yeast-network	**obs**	**null mean**	**lower CI**	**upper CI**	**t**	**p**
connectance	0.51	0.58	0.57	0.59	16.93	<0.001
links per species	2.00	2.28	2.24	2.32	16.93	<0.001
cluster coefficient	0.47	0.67	0.63	0.70	12.33	<0.001
weighted nestedness	0.37	0.54	0.46	0.61	5.00	<0.001
interaction strength asymmetry	-0.19	-0.12	-0.13	-0.11	14.76	<0.001
specialisation asymmetry	0.33	0.29	0.26	0.32	-2.93	0.017
interaction evenness	0.71	0.75	0.75	0.75	51.19	<0.001
H'_2	0.20	0.06	0.06	0.07	-51.19	<0.001
cluster coefficient HL (poll)	0.57	0.72	0.70	0.73	23.81	<0.001
cluster coefficient LL (yeasts)	0.83	0.86	0.85	0.88	5.64	<0.001
togetherness HL (poll)	0.33	0.32	0.31	0.34	-1.34	0.213
togetherness LL (yeasts)	0.15	0.21	0.19	0.22	7.82	<0.001
C.score HL (poll)	0.20	0.16	0.13	0.18	-3.27	0.010
C.score LL (yeasts)	0.45	0.24	0.20	0.27	-13.46	<0.001
robustness HL (poll)	0.73	0.76	0.71	0.80	1.26	0.241
robustness LL (yeasts)	0.92	0.93	0.91	0.95	1.34	0.213
generality HL (poll)	7.03	8.34	8.27	8.42	40.65	<0.001
vulnerability LL (yeasts)	2.89	3.41	3.38	3.43	42.11	<0.001

Appendix Table 12: Bipartite interaction indices (Dormann et al., 2008) for yeast species from yeast-pollinator-network (Fig. 10b). Singletons were not considered in this analysis (D. hansenii, D. marama, *F. oeirensis, F. chernovii*, *F. wieringae*). *

yeast species ~ pollinators	species strength	nestedrank	PDI	species specificity index	resource range	partner diversity	effective partners	d
M. reukaufii	1.23	0.00	0.72	0.00	0.45	1.15	3.15	0.03
A. pullulans	0.33	0.28	0.73	0.25	0.44	1.17	3.21	0.05
P. laurentii	0.61	0.17	0.85	0.25	0.55	1.06	2.88	0.19
M. gruessii	0.68	0.06	0.68	0.00	0.43	1.18	3.25	0.02
V. victoriae	0.11	0.50	0.92	0.75	0.73	0.56	1.75	0.29
C. rancensis	0.32	0.22	0.70	0.25	0.48	1.08	2.95	0.04
M. pulcherrima clade	0.51	0.11	0.77	0.25	0.46	1.15	3.17	0.06
S. metaroseus	0.29	0.39	0.88	0.50	0.61	0.87	2.38	0.38
S. bombicola	0.27	0.44	0.92	0.75	0.73	0.56	1.75	0.30
C. bombi	0.23	0.33	0.83	0.25	0.51	1.09	2.98	0.03
M. kunwiensis	0.16	0.56	0.92	0.75	0.73	0.56	1.75	0.32
H. takashimae	0.04	0.61	0.92	0.75	0.73	0.56	1.75	0.08
D. hungarica	0.04	0.67	1.00	1.00	1.00	0.00	1.00	0.31
D. cannabinae	0.04	0.72	1.00	1.00	1.00	0.00	1.00	0.31

*Considering overall incidence of yeast species *S. metaroseus* (d=0.38), *M*. kunwiensis (d=0.32), *D. hungarica*, *D. cannabinae* (both d=0.31), *S. bombicola* (d=0.30) and *V. victoriae* (d=0.29) are most specialized on specific pollinator groups. *M. gruessii* (d=0.02), M. reukaufii, C. bombi (both d=0.03), *C. rancensis* (d=0.04) and *A. pullulans* (d=0.05) were least specialized. Partner (Shannon-) diversity was highest for *M. gruessii* with 1.18, *A. pullulans* with 1.17, *M. reukaufii* with 1.15 and *M. pucherrima* clade with 1.15. Highest effective partners were found for *M. gruessii*, too, with 3.25, followed by *A. pullulans* with 3.21, *M. pulcherrima* clade with 3.17 and *M. reukaufii* with 3.15.

Appendix Table 13: Results of t-tests between observed bipartite network indices (Dormann et al., 2008) of the plant-yeast-network (Fig. 10c) and the average of ten null-models. "obs" is the observed value from the original network, "null mean" is the average value from the ten null-models, "lower CI" and "upper CI" give the lower and upper limit of the 95% confidence interval. Weighted nestedness (p=0.181) was not significant different, because of the small number of only 3 different tree species.

plant-yeast-network	obs	null mean	lower CI	upper CI	t	P
connectance	0.52	0.76	0.74	0.77	28.95	<0.001
links per species	1.17	1.70	1.66	1.74	28.95	<0.001
cluster coefficient	0.44	0.76	0.72	0.79	21.00	<0.001
weighted nestedness	0.39	0.18	-0.15	0.50	-1.45	0.181
interaction strength asymmetry	-0.33	-0.18	-0.20	-0.15	15.74	<0.001
specialisation asymmetry	0.30	0.32	0.26	0.37	0.55	0.594
interaction evenness	0.68	0.79	0.79	0.80	98.25	<0.001
H'_2	0.50	0.05	0.04	0.06	-98.25	<0.001
cluster coefficient HL (plants)	0.67	0.81	0.79	0.82	20.73	<0.001
cluster coefficient LL (yeasts)	0.69	0.97	0.95	0.98	44.61	<0.001
togetherness HL (plants)	0.17	0.32	0.28	0.36	8.93	<0.001
togetherness LL (yeasts)	0.44	0.07	0.04	0.10	-28.03	<0.001
C.score HL (plants)	0.67	0.35	0.25	0.45	-7.11	<0.001
C.score LL (yeasts)	0.25	0.43	0.28	0.58	2.74	0.023
robustness HL (plants)	0.54	0.70	0.68	0.72	17.34	<0.001
robustness LL (yeasts)	0.69	0.94	0.92	0.95	37.35	<0.001
generality HL (plants)	3.91	5.41	5.36	5.46	64.52	<0.001
vulnerability LL (yeasts)	1.72	2.41	2.39	2.43	82.03	<0.001

Appendix Table 14: Bipartite interaction indices (Dormann et al., 2008) for yeast species from yeast-plant-network (Fig. 10c). Singletons were not considered in this analysis (S. metaroseus, S. strictum). **

yeast species ~ plants	species strength	nestedrank	PDI	species specificity index	resource range	partner diversity	effective partners	D
M. reukaufii	0.90	0.00	0.52	0.40	0.00	0.90	2.46	0.08
A. pullulans	0.46	0.13	0.88	0.73	0.50	0.49	1.63	0.12
P. laurentii	0.30	0.50	1.00	1.00	1.00	0.00	1.00	0.32
M. gruessii	0.40	0.25	0.82	0.65	0.50	0.58	1.78	0.22
V. victoriae	0.84	0.38	0.96	0.89	0.50	0.26	1.29	0.80
B. rancensis	0.05	0.63	1.00	1.00	1.00	0.00	1.00	0.14
M. pulcherrima clade	0.03	0.75	1.00	1.00	1.00	0.00	1.00	0.09

**Considering overall incidence of yeast species *V. Victoriae* (d=0.80) is highly specialized on single tree species, followed by *P. laurentii* (d=0.32), whereas *M. reukauffi* (d=0.08) and *M. pulcherrima* clade (d=0.09) were least specialized. Partner (Shannon-) diversity was highest for *M. reukaufii* with 0.90, followed by *M. gruessii* with 0.58, *A. pullulans* with 0.49 and *V. victoriae* with 0.26. *M. reukaufii* showed highest effective partners, too, with 2.46, followed by *M. gressii* with 1.78, A. pullulans with 1.63 and *V. victoriae* with 1.29.

Appendix Table 15: Species indicator analysis with absolute abundance of yeast species isolated from pollinators per single observation as indicator species (19 species) and source insect group as site groups (5 groups: bumblebees, honeybees, wild bees, sawflies, wasps). Group Combinations are allowed. Only results with $p<0.05$ are shown. n=175 observations. "target" gives the probability that the surveyed yeast species belongs to the target insect group, "other" to the other insect groups and "overall" to all tested insect groups together. "p-value" is given for "target". No indicator species was found for Wasps.

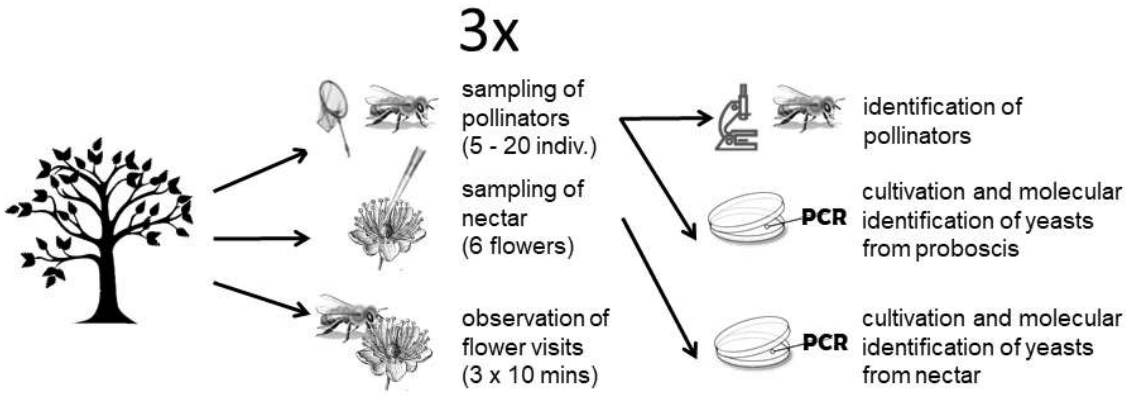
Species	target	other	overall	p-value
Bumblebees				
Metschnikowia pulcherrima clade	0.526	0.229	0.347	0.027
Sawflies				
Sporobolomyces metaroseus	0.886	0.114	0.318	0.016
Bumblebees + Honeybees + Wild bees				
Metschnikowia reukaufii	0.923	0.476	0.663	0.001
Metschnikowia gruessii	0.971	0.343	0.577	0.001
Candida rancensis	0.925	0.133	0.351	0.046

Appendix Table 16: Species indicator analysis with absolute abundance of yeast species isolated from nectar per single observation as indicator species (9 species) and tree species as site groups (3 groups). Group Combinations are allowed. Only results with $p<0.05$ are shown. n=35 observations. "target" gives the probability that the surveyed yeast species belongs to the target tree species, "other" to the other groups and "overall" to all tested groups together. "p-value" is given for "target".

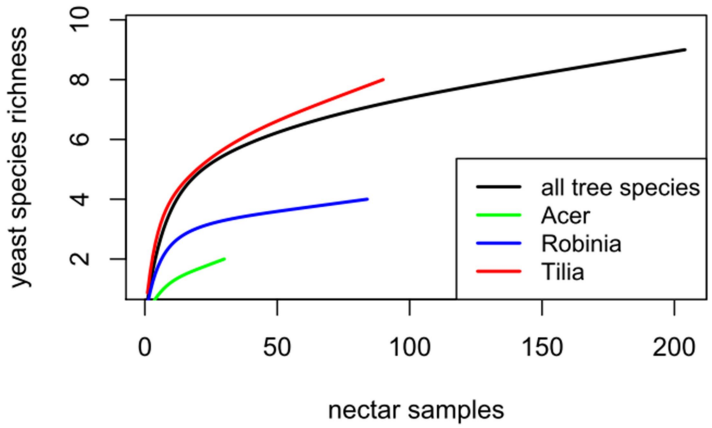
Species	target	other	overall	p-value
Acer platanoides				
Vishniacozyma victoriae	0.921	0.500	0.679	0.010
Tilia platyphyllos				
Papiliotrema laurentii	1.000	0.667	0.816	0.002
Aureobasidium pullulans	0.796	0.733	0.764	0.004
Candida rancensis	1.000	0.400	0.632	0.007
Robinia pseudoacacia + Tilia platyphyllos				
Metschnikowia reukaufii	0.9375	0.621	0.763	0.034

Figures

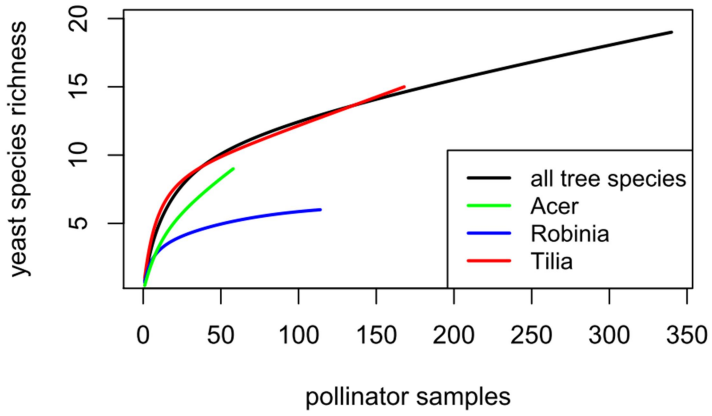
Appendix Figure 4: Location of sampled trees in the Uckermark region. Five individual trees each of three different tree species were sampled. Acer platanoides (green dots), Robinia pseudoacacia (yellow dots) and Tilia platyphyllos (red dots).



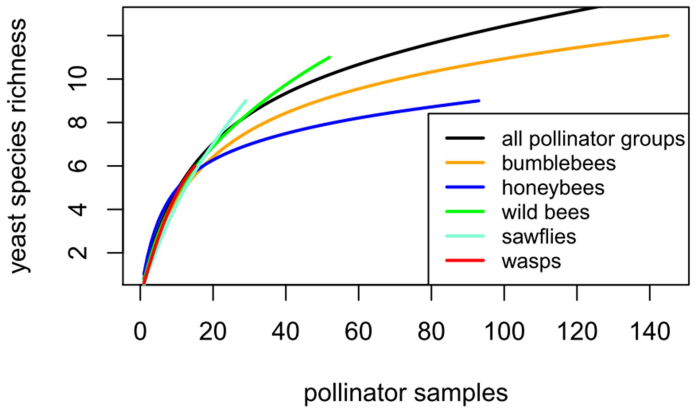
Appendix Figure 5: Overview of the sampling procedure. Each tree was sampled three times; sampling included catching pollinators, taking nectar samples and observation of pollinator visitation frequency. Pollinator and nectar samples were analyzed for yeast species.



Appendix Figure 6: Species accumulation curve of yeast species richness from nectar samples for all samples and subsamples from the tree species *Acer, Robinia* and *Tilia*. Curves are calculated with the R package "vegan" function "specaccum". 70 flowers harbored only one yeast species, 24 flowers contained two species and 3 flowers had 3 species.



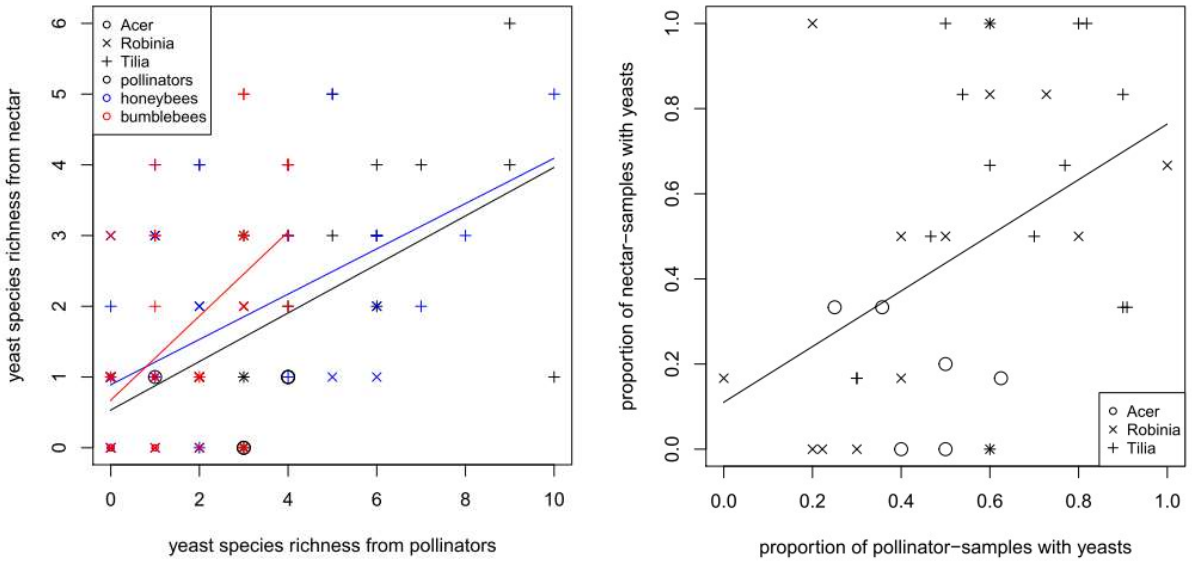
Appendix Figure 7: Species accumulation curve of yeast species richness isolated from pollinator samples for all samples and subsamples from the tree species *Acer, Robinia* and *Tilia*. Curves are calculated with the R package "vegan" function "specaccum". 119 pollinators transported only one fungal species, 59 pollinators transported two species, 10 pollinators transported three species and one pollinator transported four species.



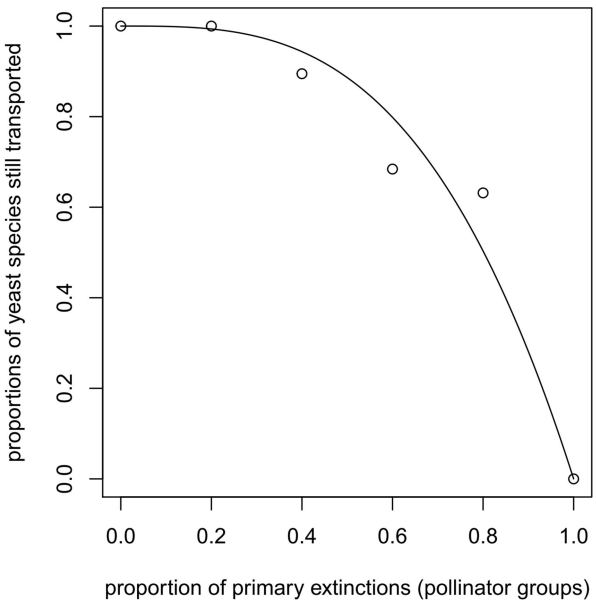
Appendix Figure 8: Species accumulation curve of yeast species richness isolated from pollinator samples for all samples and subsamples from the insect groups bumblebees, honeybees, wild bees, sawflies and wasps. Curve for all pollinator groups is not completely drawn (see Appendix Fig. 7). Curves are calculated with the R package "vegan" function "specaccum". 119 pollinators transported only one fungal species, 59 pollinators transported two species, 10 pollinators transported three species and one pollinator transported four species.

a)

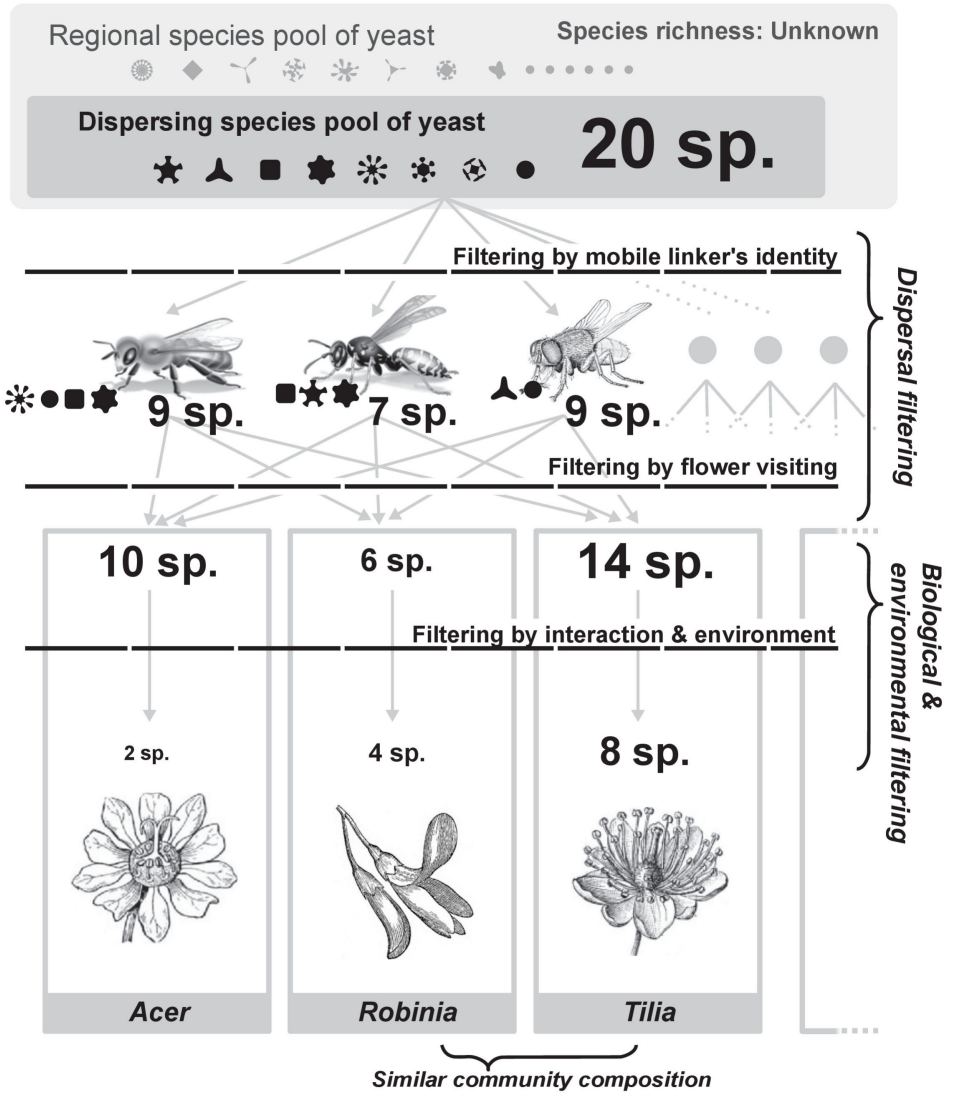
b)



Appendix Figure 9: Relationships between yeast species richness found in nectar against species richness isolated from pollinators (a): Proportion of nectar-samples containing at least one yeast species against same proportion of pollinator samples (b). Data points represent single observations (n=35) at Acer (circles), Robinia (crosses) and Tilia (plusses). Black data points refer to all pollinator groups, blue data points represent honeybee-data and red data points bumblebee-data. Results from statistical tests are given in appendix Table 10.



Appendix Figure 10: Second extinction curve of yeast-pollinator-network (Fig 10b): Proportion of primary extinctions of pollinator groups (n=5) against proportion of yeast species still transported in the network (n=19). Yeast diversity transported by pollinators remains at 89.5% (compared to the complete network) even if two of five (40%) of pollinator groups are randomly excluded from the network.



Appendix Figure 11: Graphical abstract: The authors describe yeasts species pools transported by pollinator guilds and compare those with yeast metacommunities in floral nectar, which allows them to draw conclusions on effective dispersal pathways of different yeast groups and identify the most important local and regional processes that shape the structure of the yeast metacommunities.

B: Molecular yeast identification (Method)

Yeast colonies from incubated agar plate samples were transferred to single agar plates for isolation and grown again for 5 days at 25°C in the dark. Yeasts were morphologically sorted into different morphotypes. 25% randomly chosen samples of one morphotype were identified genetically. If all subsamples were from the same species all other samples from this morphotype were expected to be the same species. If subsamples showed different species all other samples of the morphotype were identified. Amplification was performed based on the D1/D2 domains of the large subunit nuclear ribosomal RNA following Lachance et al. (1999). For molecular identification cell material was taken from an agar plate and added to a 50 µl Master Mix made of 10 µl Firepol Master Mix (Solis Biodyne, Tartu, Estonia), 36 µl water, 2 µl primer NL1 (5′-GCA TATCAA TAA GCG GAG GAA AAG-3′) and 2 µl primer NL4 (5′-GGT CCG TGT TTC AAG ACGG-3′). The PCR temperature profile consisted of an initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 55°C for 10 s and 72°C for 20 s, and a final extension at 72°C for 1 min. PCR products were examined by agarose gel electrophoresis and quantified using a Nano Photometer (Implen, München, Germany). Afterwards we purified the PCR products using a PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Obtained amplicons were sequenced by LGC Genomics (Berlin, Germany) using the same reverse primers as those used for amplification. The sequences were aligned and clustered into operational taxonomic units (OTUs) with the MAFFT algorithm (MAFFT Version 7). Similar sequences were grouped and realigned. Single sequences from the resulting clades were identified with Mycobank database (Robert et al. 2005) using pairwise sequence alignment. Species identification was accepted if similarity was 99% or more. A group of species was combined to the M. pulcherrima clade (Guzmán et al. 2013). A subset of samples was stored at -80 ° C in YM broth containing 15% glycerol. At least one strain per yeast species was deposited at Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures under accession numbers DSMZ105599 to 105624. Yeast systematics follows Liu et al. (2015) and Wang et al. (2015).

C: Drivers of flower visits

Materials and Methods

Landscape analysis

The proportion of Biotope habitat classes within a radius of 250m from the sampled trees was calculated based on the data set BTLN Brandenburg (LUGV 2009) following (Tonietto et al. 2011). Habitats were separated into the following classes: field, ruderal, building, waterbody, green -area, shrub, swamp, forest and other. Geographical analyses were done with the soft-ware ArcMap 10.4.1 (Environmental Systems Research Institute Inc., 2017).

Machine learning

The frequencies of flower visits by the different insect groups were modelled individually against landscape structure measurements, using the random forest algorithm with variable se-lection (Breimann 2001; Hapfelmeier & Ulm, 2013) that can conduct a non-parametric multiple regression (e.g. Bergmann, Ryo, Prati, Hempel, & Rillig, 2017; Ryo, Yoshimura, & Iwasaki, 2017; Ryo and Rillig, 2017). We estimated the relative contribution of each predictor to explain-ing the variability in the response based on model fit measured by the coefficient of determina-tion R2 (Breiman 1996). We used the 'party' package (version 1.2.3; Strobl et al., 2009). In hy-per-parameter settings, the number of trees was set to 1000, and the number of permutation for estimating statistical significance was set to 4000. We employed partial dependence plots (Has-tie et al., 2009) for visualizing the modelled associations. The higher the value, the higher the visit frequency.

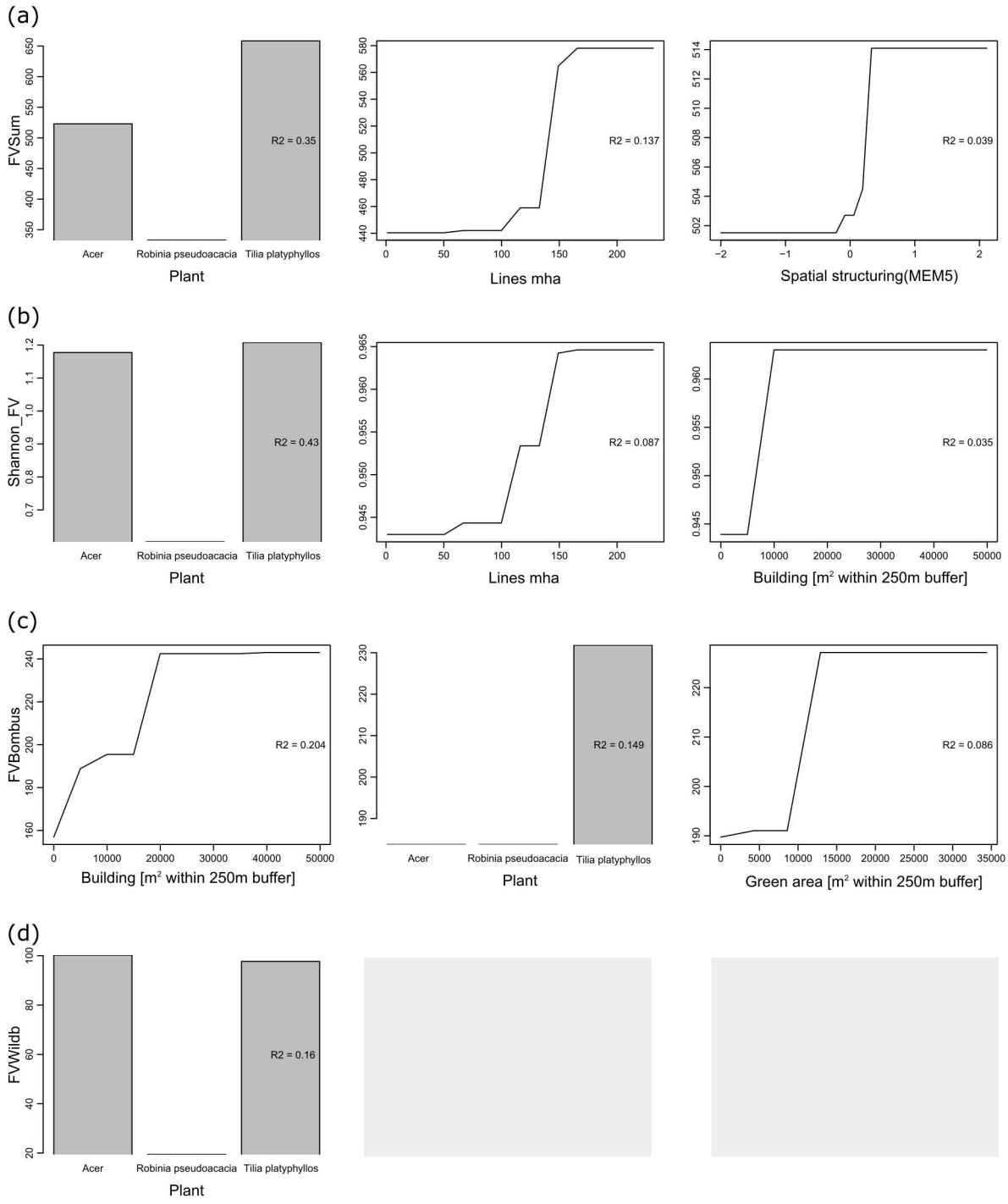
Results

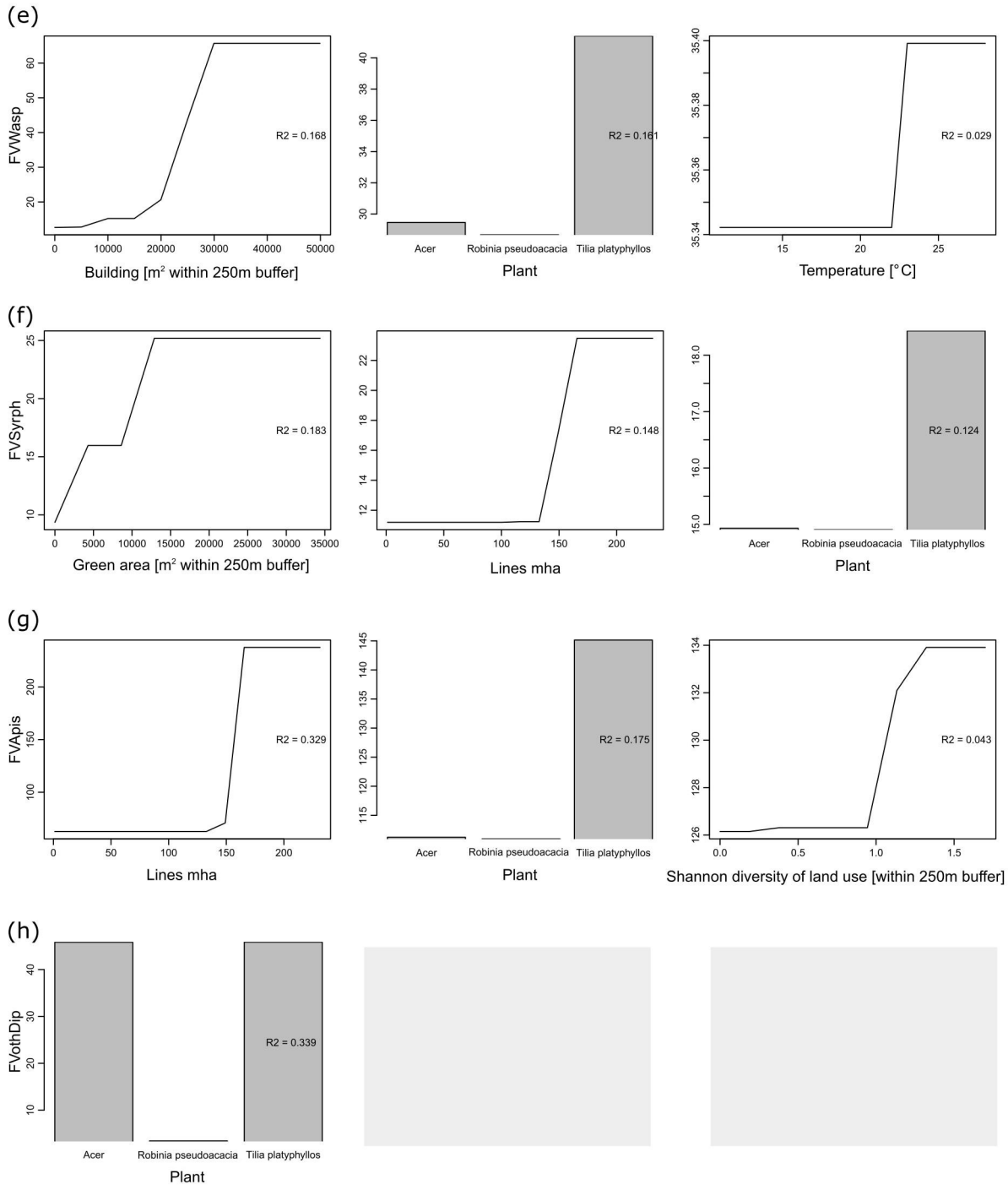
Flower visits of pollinators explained yeast species richness and abundance in flowers. We analyzed how landscape composition affects pollinator density and therefore has an indirect effect on the yeast meta-community. Results of machine learning testing flower visits of different in-sect groups against landscape structure measurements are given in appendix Table 17 and appendix Fig. 12. Total flower visits were best explained by tree species (R^2=0.35), biotope structure (R^2=0.14) and spatial distribution (R^2=0.04). Flower visits of honeybees were driven by biotope structure (R^2=0.33), tree species

(R^2=0.18) and Shannon-diversity of biotopes (R^2=0.04). Flower visits of bumblebees was best predicted by proportion of buildings (R^2=0.20), tree species (R^2=0.15) and proportion of green area (R^2=0.09).

Appendix Table 17: Results of machine learning using random forest analysis of flower visits of different insect groups and Shannon diversity of pollinator groups against abiotic factors, spatial autocorrelation of sampling locations (MEM5), proportion of biotope types (n=12) in 250m radius around sampling locations, shannon-diversity of biotope types and landscape structure as biotope lines and borders in m/ha in the same radius. Values represent R^2 fitting. Only relevant independent variables are listed.

Dependent variables	**R^2 sum (fitting)**	**tree spe-cies**	**buil-dings**	**green-area**	**Bio-tope struc-ture**	**Shan-non-div. bio-topes**	**Tempe-rature**	**MEM 5**	**field**
Flower visits sawflies	0.56	0.124	0.066	0.183	0.148	0.001	0.033	0	0
Flower visits other flies	0.34	0.339	0	0	0	0	0	0	0
Flower visits beetles	0.00	0	0	0	0	0	0	0	0
Flower visits honeybees	0.63	0.175	0.007	0.032	0.329	0.043	0.028	0.02	0
Flower visits bumblebees	0.50	0.149	0.204	0.086	0	0.014	0	0	0.051
Flower visits wild bees	0.17	0.16	0	0	0	0	0.008	0	0
Flower visits wasps	0.36	0.161	0.168	0	0	0	0.029	0	0
Flower visits sum	0.58	0.35	0.031	0.026	0.137	0	0	0.039	0
Shannon diversity of flower visits of insect groups	0.62	0.43	0.035	0.03	0.087	0.034	0	0	0


(a)
FVSum
R2 = 0.35
Acer
Robinia pseudoacacia
Tilia platyphyllos
Plant
R2 = 0.137
Lines mha
R2 = 0.039
Spatial structuring(MEM5)
(b)
Shannon_FV
R2 = 0.43
Acer
Robinia pseudoacacia
Tilia platyphyllos
Plant
R2 = 0.087
Lines mha
R2 = 0.035
Building [m² within 250m buffer]
(c)
FVBombus
R2 = 0.204
Building [m² within 250m buffer]
R2 = 0.149
Acer
Robinia pseudoacacia
Tilia platyphyllos
Plant
R2 = 0.086
Green area [m² within 250m buffer]
(d)
FVWildb
R2 = 0.16
Acer
Robinia pseudoacacia
Tilia platyphyllos
Plant



Appendix Figure 12 (a – h): Plotted results of machine learning using random forest analysis of flower visits of different insect groups and Shannon diversity of pollinator groups against abiotic factors, spatial distribution of sampling locations (MEM5), proportion of biotope types (n=12) in 250m radius around sampling locations, Shannon-diversity of biotope types and landscape structure as biotope lines in m/ha in the same radius. Only three independent variables with highest R^2-value are plotted.

Discussion

After differences in tree species the number of flower visits of all pollinators was best explained by higher landscape structure. Therefore, we conclude pollinators do not depend on a single habitat type or a high diversity of biotopes, but on highly structured landscapes with many ecotones. Even if honeybee density would be expected to be higher near villages due to human beekeeping, it was best predicted by landscape structure, too. Surprisingly, bumblebees were best predicted by higher proportion of buildings and green area, which is correlated with villages. This may be caused by a higher availability of cavities used for nesting in gardens with old trees and old buildings.

References (Appendix 2)

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