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Research Article

RESEARCH ARTICLE

Solving the puzzle of yeast survival in ephemeral nectar systems: exponential growth is not enough

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One sentence summary: The authors examine how nectar yeasts disperse and how their populations develop cell densities sufficient to have ecological effects when faced with regular decimation by pollinator visits.

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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.

Keywords: nectar yeast; dispersal; pollination; growth rate; movement ecology

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

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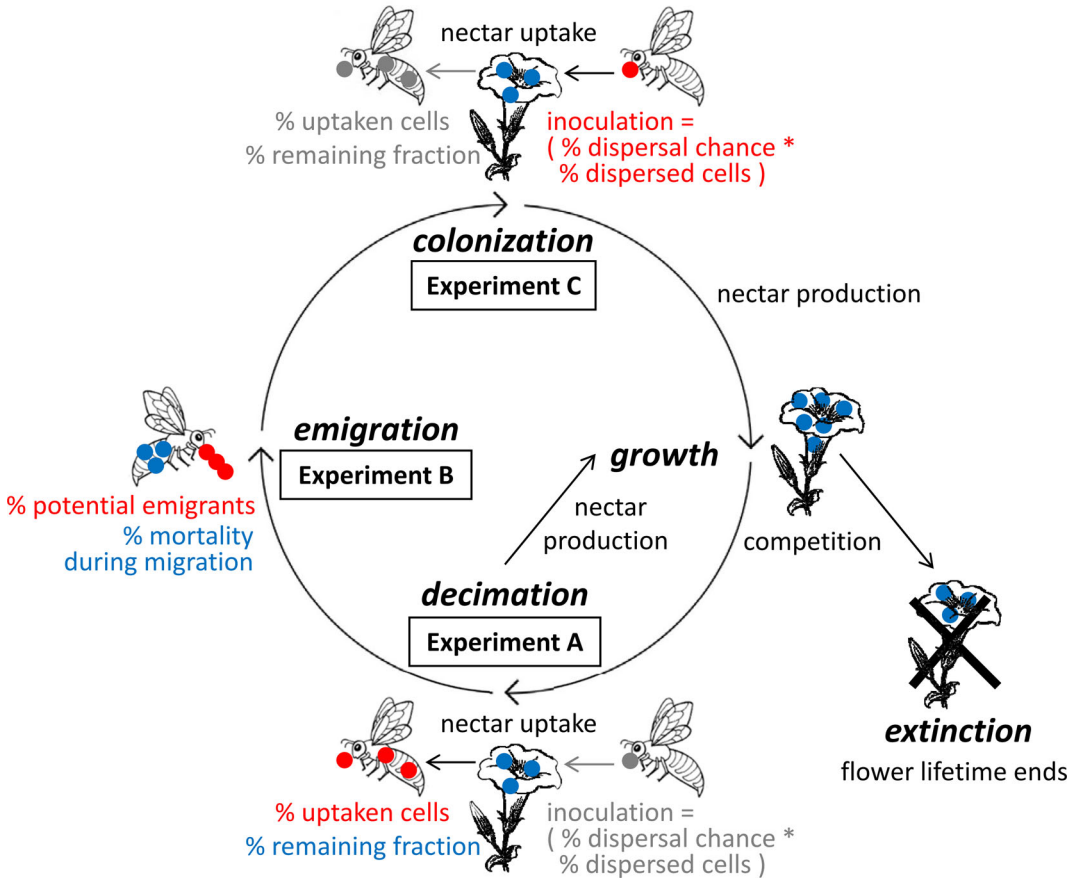


Figure 1. Simplified depiction of the movement ecology of nectar yeasts with a focus on dispersal by pollinators. The black bold italic terms are main steps in the lifecycle, the terms in black regular font are processes, and the variables in red and blue were measured in our experiments. Terms in frames 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.

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. 1). 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.

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.

MATERIALS AND METHODS

Before planning the lab experiments, we conducted a sensitivity analysis (see supplementary material A) 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. 2). 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.

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

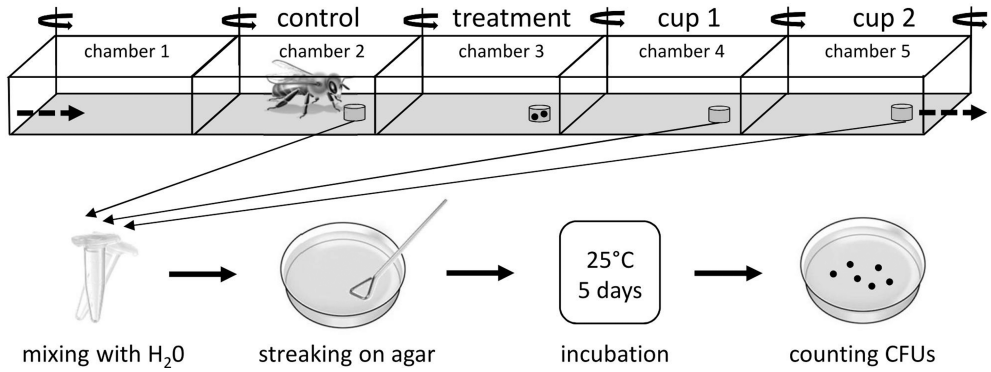


Figure 2. Design of experiment C to measure proportion of dispersed nectar yeast cells. Honeybees passed the tunnel and drank 10 μl artifical nectar each in the chambers 2 to 5. In chamber 3, yeasts were added to the nectar. Remaining cells from the cups were mixed with water, streaked on agar and incubated,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

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). '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 al* 2015, 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.

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.

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.

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	10 948	<0.001
	Density	1	88	10 784	<0.001
	Cup	1	87	10 777	0.012
	Species: density	1	86	10 713	<0.001
	Species: cup	1	85	10 137	<0.001
	Density: cup	1	84	10 137	0.438
	Species: density: cup	1	83	9971	<0.001

'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.

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.

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 ($P < 0.001$).

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

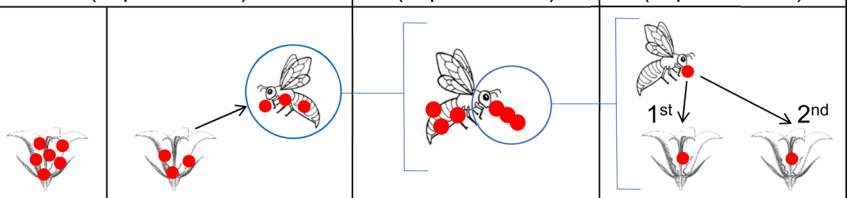
	cells in flower (experiment A)			transported cells (experiment B)		cells dispersed (experiment C)	
							
Metschnikowia reukaufii	total	remaining	uptaken	swallowed	on proboscis	1st flower 71.1% chance	2nd flower 33.3% chance
absolute cell number	10 000	4120	5880	5565	315	17	10
fraction of cells in flower (%)	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 3. 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.

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.

To summarize our findings, Fig. 3 shows the absolute cell numbers and proportion of cells at different dispersal steps if the total source population is 10^4 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.

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.

The higher the 'initial cell number (source population)', the higher the resulting cell density (Fig. 4; $P < 0.001$, Table 2) and the total amount of dispersed cells (Fig. 5; $P < 0.001$, 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. 4A and B; $P < 0.001$, Table 2) and 'remaining fraction' (Fig. 4C; $P < 0.001$, 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. 4D; $P < 0.001$, Table 2). Dispersed cells also increased exponentially with higher 'growth rate' (Fig. 5A 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. 5C; $P < 0.001$, 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. 5D; $P = 0.024$, Table 2).

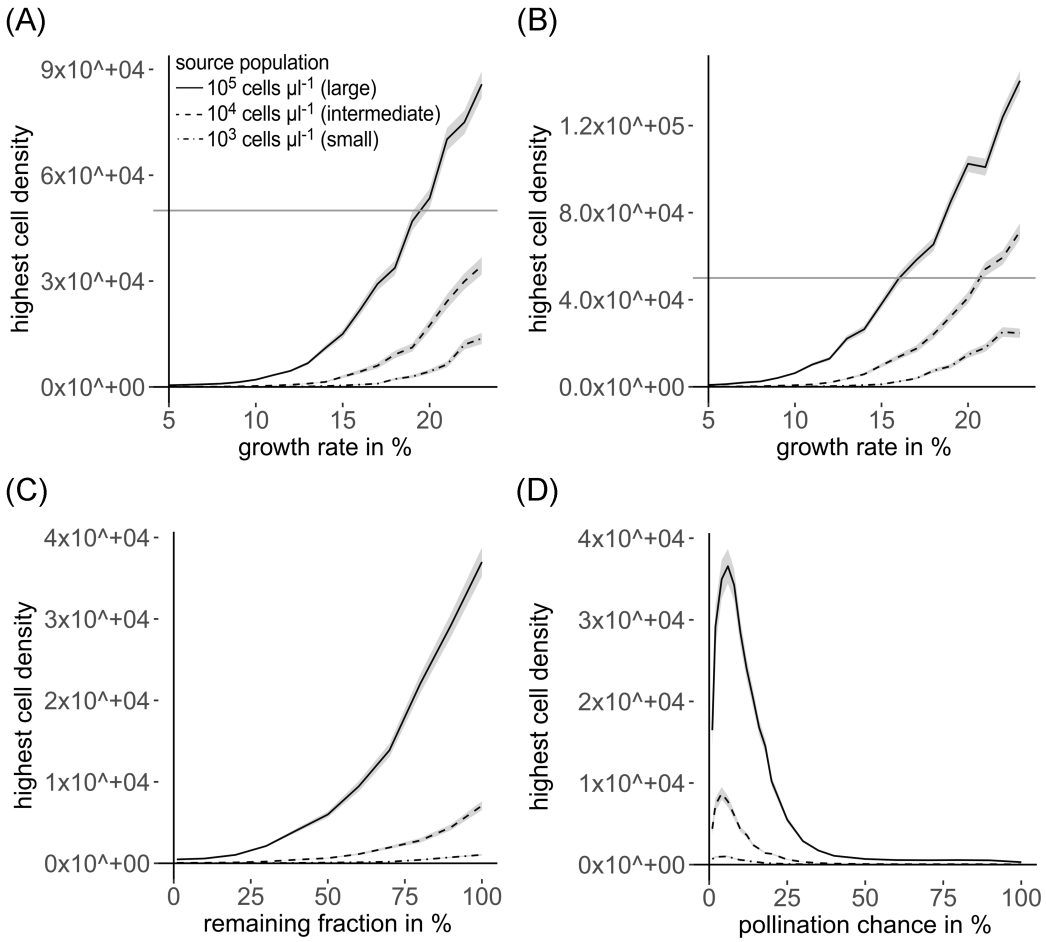


Figure 4. Results of highest cell density for simulations of different steps of growth rate (**A** and **B**), remaining fraction (**C**) and pollination chance (**D**). For A, C and D: default values for parameters that were not altered on the x-axes are 10% growth rate, 30% remaining fraction and 33% 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.

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}).

Dependent variable	Independent variables	Num d.f.	Den d.f.	Dev.	*P* value
Highest cell density	Growth rate	1	221 998	2.8231e + 15	<0.001
	Remaining fraction	1	221 997	2.7516e + 15	<0.001
	Pollination chance	1	221 996	2.6620e + 15	<0.001
	Source population	1	221 995	2.2968e + 15	<0.001
	Growth rate: remaining fraction	1	221 994	2.2967e + 15	0.522
	Growth rate: source population	1	221 993	2.2683e + 15	<0.001
	Remaining fraction: source population	1	221 992	2.2666e + 15	< 0.001
	Pollination chance: source population	1	221 991	2.2660e + 15	<0.001
	Growth rate: remaining fraction: source population	1	221 990	2.2654e + 15	<0.001
Total dispersed cells	Growth rate	1	221 998	5095 954 884	<0.001
	Remaining fraction	1	221 997	5094 350 498	<0.001
	Pollination chance	1	221 996	5085 424 596	<0.001
	Source population	1	221 995	4383 567 883	<0.001
	Growth rate: remaining fraction	1	221 994	4382 035 372	<0.001
	Growth rate: source population	1	221 993	4359 520 760	<0.001
	Remaining fraction: source population	1	221 992	4354 281 131	<0.001
	Pollination chance: source population	1	221 991	4353 924 127	0.024
	Growth rate: remaining fraction: source population	1	221 990	4353 924 125	0.995

Num d.f.: numerator degrees of freedom; Den d.f: denominator degrees of freedom.

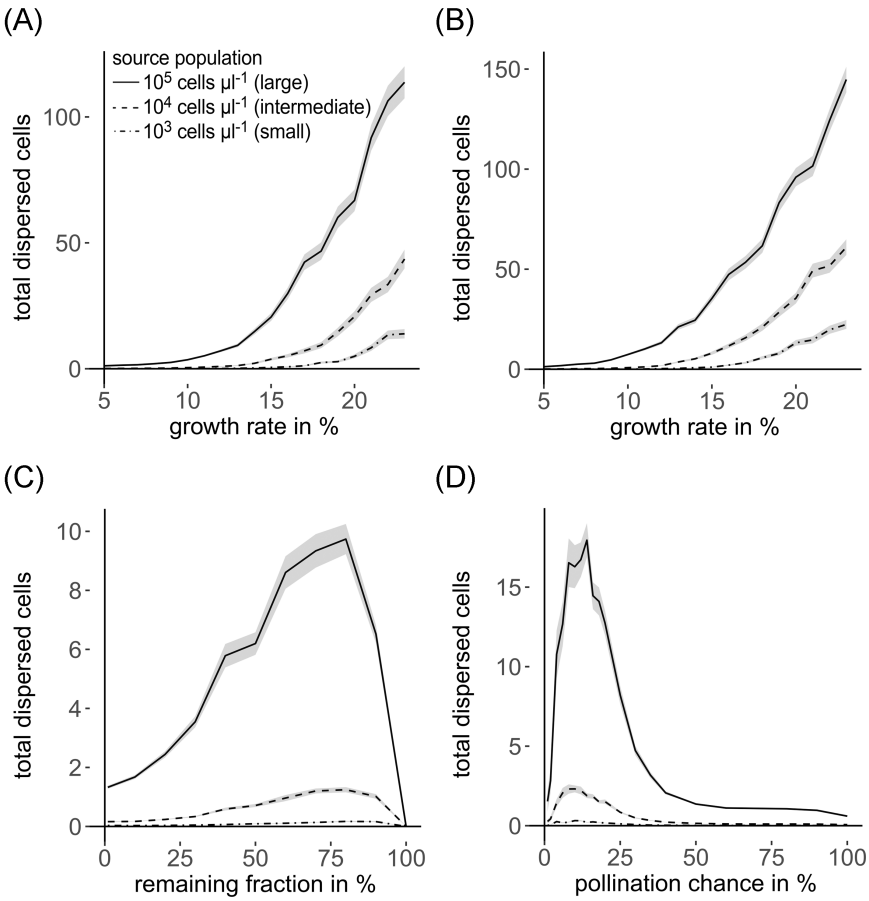


Figure 5. Results of total dispersed cells for simulations of different steps of growth rate (**A** and **B**), remaining fraction (**C**) and pollination chance (**D**). For A, C and D: default values for parameters that were not altered on the x-axes are 10% growth rate, 30% remaining fraction and 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.

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. 4A). 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. 4B).

The target cell density cannot be reached at any level of 'remaining fraction' with default values (10% 'growth rate', 33% 'pollination chance', Fig. 4C). 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).

Population size does not reach target cell density for any 'pollination chance' level with default values (10% 'growth rate', 30% 'remaining fraction', Fig. 4D). 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}.

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. 5C) 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

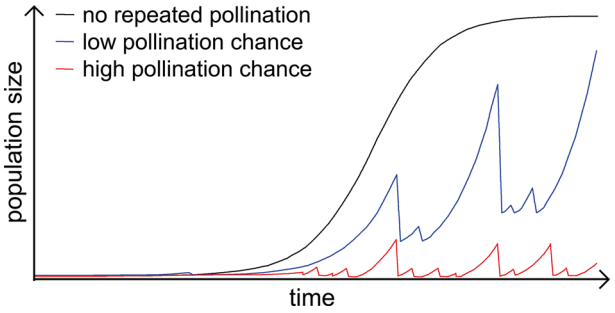


Figure 6. Schematic development of population size of a local nectar yeast colony over time with just one pollination event in the beginning (black line), low supporting (blue line) and too high hindering (red line) pollination chance. Population decimations are caused by nectar extraction during pollination events.

shown in the conceptual Fig. 6. 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.

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-Table S4). In our sensitivity analysis (supplementary material A3), 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. 5A). 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) 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 4C 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. 3). 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.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared

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