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Variable dependency on associated yeast communities influences host range in *Drosophila* species

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The tight association between yeast metabolites and the attraction of fly species provides key evolutionary innovations that generate immense diversity within the genus *Drosophila*. Why and how changes in *Drosophila* niche preferences occur, and what role yeasts play in species specialization, is still largely unknown. Here, we analyze adult preference and larval development across three species of *Drosophila* as well as niche modifications through joint efforts by both insect and yeast. In general, we found that niche specialization is more a result of larval constraints than adult oviposition choice. We also determined that different life stages of fly development vary in their respective yeast preference, which may reduce cross-generational competition for resources. In this way, natural selection pressures may act quite differently on adult or larval performance, with adaptation events in larval stages likely being the stronger driver of niche evolution. Furthermore, we provide evidence that *Drosophila* and yeast together process host material to promote mutual benefits. Our data indicate that adult *Drosophila* flies of different species are flexible in their yeast preference and can be attracted to yeast species from radically different habitats. This argues against stable *Drosophila*-yeast associations and for broader acceptance of diverse yeast species across adult individuals in the genus *Drosophila*.

Keywords: cross-generation competition, *Drosophila*–yeast interaction, mutualism, niche construction, speciation, tritrophic

Introduction

What drives host (food source and breeding site) preference across the genus *Drosophila*? Does the association of *Drosophila* species with certain habitats arise from benefits in fitness for the flies themselves and how does the nature of their associated microorganisms determine the host preference of each fly? Studying the principles and mechanisms underlying the odor-mediated interaction of *Drosophila* flies with yeast species that share the same habitat and ecological niche allows for insights into the evolution of mutualism and niche specialization. It is believed that metabolic activities of a sessile organism, together with physical modifications of its habitat,

have the potential to create an ecological niche (also known as niche construction), which in return affects other organisms living in the same environment (Bertness 1984, Huntly and Inouye 1984, Facelli et al. 1991, Morawetz et al. 1992, Laland et al. 1999, Odling-Smee et al. 2003, 2013, Goddard 2008, Buser et al. 2014). Moreover, organisms populating the same habitat as a niche constructor may experience benefits to their reproductive success through these habitat modifications (Odling-Smee et al. 2013). In this case, chemical and visual cues that originate from the niche constructor can become mediators of an interaction between constructor and beneficiary. Thus, what may have started on an evolutionary timescale as a one-way interaction between beneficiary (insect) and niche constructor (yeast) can instead evolve into an interspecies mutualism if the niche constructor in return also profits from an association with the beneficiary (Doebeli and Knowlton 1998, Herre et al. 1999, Odling-Smee et al. 2013, Buser et al. 2014).

A prominent example for niche construction is the release of ethanol, heat and CO_2 into the surrounding environment by the fruit-associated yeast *Saccharomyces cerevisiae* as part of the yeast's energy production via fermentation of sugars found in its host material (i.e. growth substrate, Goddard 2008). The combination of these niche modifications can suppress the growth of other microorganisms and secure essential resources for *S. cerevisiae* by creating a competitive advantage (Goddard 2008). We speculate that in much the same way, the presence of *S. cerevisiae* at breeding sites of *Drosophila* species could prevent microorganisms that are harmful for adult and larval life stages to establish within the flies' food source (e.g. pathogens and fungal molds).

The interactions between *Drosophila* flies and yeasts have been shown to be mediated by odors, as is the interaction of many other insects with yeasts (Witzgall et al. 2012, Davis et al. 2013, Andreadis et al. 2015, Madden et al. 2018, Pozo et al. 2019).

In the case of *D. melanogaster*, emission of volatiles by associated yeasts has been proven to be a key factor in the flies' attraction and preference towards a substrate as a food source or oviposition site. Moreover, that odorants released by the host substrate itself (in this case decaying fruit or vegetable material) play only a secondary role in this attraction behavior (Becher et al. 2012, Scheidler et al. 2015). Furthermore, recent findings show that the presence of viable yeast cells in fly diet causes changes in larval development and survivorship as well as changes in adult phenotypic traits (e.g. fecundity and cuticular hormone production), adult behavior (e.g. food choice) and longevity (Anagnostou et al. 2010a, Fischer et al. 2017, Bellutti et al. 2018, Grangeteau et al. 2018, Murgier et al. 2019). In many cases, yeasts enrich the flies' host substrate with nutritional factors such as amino acids, antioxidants, fatty acids, sterols and vitamin B's, which are all essential dietary components that the host alone cannot provide (Loeb and Northrop 1916, Tatum 1939, Becher et al. 2012, Dweck et al. 2015). In addition, these yeast-produced nutrients affect the ability of *Drosophila* flies to subsequently resist pathogens and parasitoids (Vass and Nappi 1998, Rivera et al. 2003, Li et al. 2007, Lee et al. 2008, Anagnostou et al. 2010b).

In exchange for nutrients supplied by yeasts, *Drosophila* flies act as a vector for the dispersal of these microbial species, often carrying yeast spores on their body or transferring them to new substrates through the oral–fecal route (Ganter 1988, Starmer et al. 1988, Reuter et al. 2007, Coluccio et al. 2008). In addition, passage through the alimentary canal of *Drosophila* flies has been shown to release yeast spores from their tetrads and enable the yeasts to sexually reproduce, which in return promotes genetic mixing (Reuter et al. 2007). In this case, sexual reproduction then allows the yeasts to adapt faster to changes in their environment through outbreeding (Reuter et al. 2007), which is similar to the advantages afforded to flowering plants via insect-driven pollination. Besides the function of dispersal and outbreeding through interaction with *Drosophila* species, previous data suggest that the presence of *D. melanogaster* larvae in host material additionally affects yeast cell numbers and the composition of yeast communities to the benefit of only a few selected yeast species (Stamps et al. 2012). Flies achieve this selective growth promotion through the deposition of frass, which contains nutritional factors and pre-selected microbes as well as through physical modifications of the host substrate structure (Stamps et al. 2012). It is known that the alimentary canal of insects as well as other invertebrates and vertebrates can act as a filtering unit for the cultivation of a selected community of microbes, including yeasts (Fig. 1A, Coluccio et al. 2008, Kakumanu et al. 2018, Stefanini 2018, Schmidt et al. 2019). We hypothesize that processes, which promote the growth of the specific beneficial yeasts of a *Drosophila* species at host locations are positively selected for as new breeding sites of *Drosophila* already possess an established microbial community before flies arrive, feed and lay their eggs (Begon 1982). In return, growth promotion and selection against other microbes by the fly allows the associated yeast to outperform other microbes occupying this ecological niche, from which both the yeast and fly profit (Stamps et al. 2012). Consequently, we expect that mutual benefits from a lifestyle in close association with each other could drive selection events in favor of these fly–yeast associations.

Evidence for mutual benefits granted to flies and yeast led to the hypothesis that the production of volatiles that are attractive to *Drosophila* flies is a yeast-specific trait, which evolved to maintain and ensure the association of yeasts with certain insects, including the genus *Drosophila*. In favor of this hypothesis it has been proposed that co-evolution of chemosignal production in yeasts accompanies the development of corresponding detecting units for these odorants by insects (Engel and Grimaldi 2004, Dujon 2012, Davis et al. 2013, Nel et al. 2013, Becher et al. 2018). Furthermore, the occurrence of metabolic pathways in yeasts that are not necessary for the microorganism's survival but lead to the production of odorants attractive to insects has been suggested as evidence for a mutualistic association of *Drosophila* and yeasts

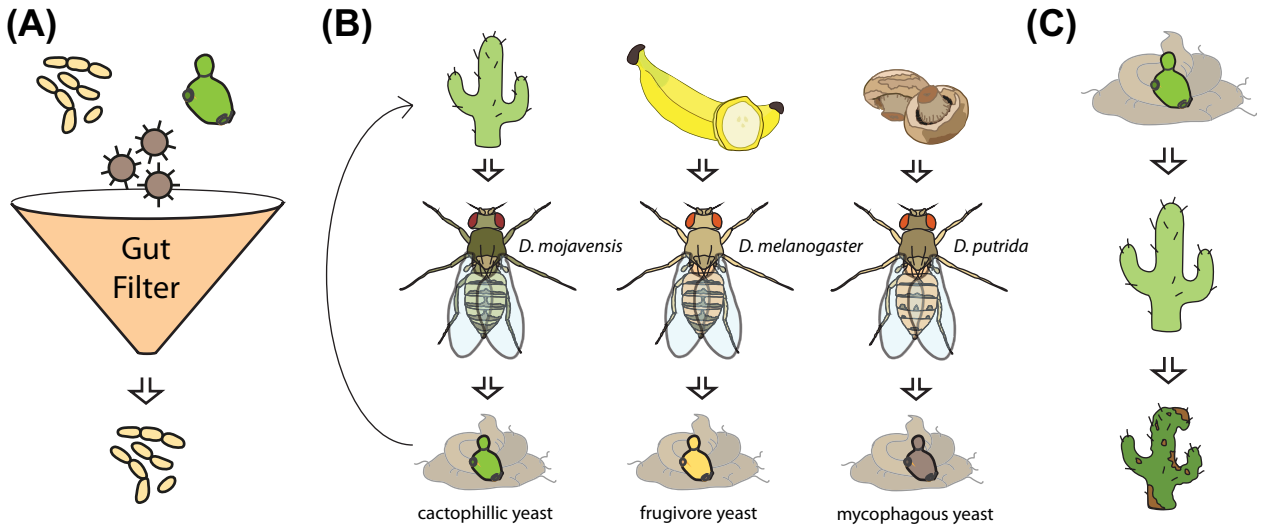

Figure 1. Hypothesized interplay of insect, yeast and their hosts. (A) A fly species-specific lifestyle and gut physiology allow only a certain set of specific microorganisms to survive and thrive in the insect's alimentary canal. Thus, the fly's gut would act as a filter putting a directed selection on ingested and finally excreted microorganisms. (B) Here, we predict that different *Drosophila* species display adaptive behaviors towards not just their host itself, but also towards yeast species associated with that ecological niche. The interaction of insect and yeast species would be mutual beneficial providing the flies with nutrition and enabling dissemination for the yeast. (C) Example of an interplay described in (A) and (B): Cactophilic *Drosophila* species is attracted to cacti and cacti-associated yeast. Yeast cells are ingested by the flies and vectored via an oral–fecal route onto new hosts. The cactophilic yeast efficiently breaks down the host material, releasing nutritional factors. We hypothesize that growth of both the insect and the yeast will be optimal when placed on cactus host material, and that this will be additionally measurable by the observed rate of host decay.

(Christiaens et al. 2014, Becher et al. 2018). For example, the production of acetyl esters by *S. cerevisiae*, such as ethyl acetate which is a highly attractive odorant for *Drosophila* flies, is mediated by an alcohol acetyl transferase encoded by the yeast's *ATF1* gene (Christiaens et al. 2014). However, the opposing hypothesis for fly–yeast interaction, postulates that yeast-volatiles are solely the by-product of the yeast's metabolic activity, for example from the detoxification of harmful fermentation intermediates, and did not evolve to maintain any insect–yeast association (Palanca et al. 2013, Günther et al. 2019). Hence, according to this hypothesis, *Drosophila* flies would have secondarily evolved to associate these chemosignals as indicators of suited host material (Palanca et al. 2013) and fly-yeast associations would form merely by chance or coincidence, through co-habitation of the same environment (Günther and Goddard 2019, Günther et al. 2019).

It is not yet known whether *Drosophila* flies actively seek out and select certain yeasts in order to acquire a species-specific microbiome that is optimized for their reproductive success. Are *Drosophila* flies farming species-specific yeast communities, transporting these yeasts to suitable substrates, and promoting the yeasts' growth while suppressing the colonization of harmful or non-beneficial microorganisms? This would be comparable to other well-studied insect–microbe partnerships such as leafcutter ants with *Lepiotaceae* fungi or the association of the European beewolf with actinobacterium '*Candidatus* Streptomyces philanthi' (Chapela et al. 1994, Kaltenpoth et al. 2005, Mueller et al. 2005, Kroiss et al. 2010, Ranger et al. 2018). In each case the microbe and insect act in a partnership with mutual benefits.

In the present study, we test the hypothesis that *Drosophila* flies will favor an association with yeast species from a shared habitat over those yeast species from an unfamiliar niche (Fig. 1B). We predict that *Drosophila* species can discriminate between yeasts based on species-specific chemosignals emitted by the yeasts, and moreover that flies will be most attracted to yeast species from a joint host substrate (for example in this study: banana, cactus or mushroom material) to ensure mutual benefits. Presumably, attractiveness of a yeast from a familiar environment would subsequently be linked to an increase in larval and adult performance. Similarly, we suspect that yeasts will also preferably attract *Drosophila* species from a shared habitat to ensure their transfer to suitable substrates for their own growth (Fig. 1C).

In agreement with previous studies, we find that *Drosophila* flies can distinguish between different yeasts based on chemosensory cues. However, we observed that *Drosophila* species may favor yeasts from new environments over yeast species from a familiar ecological niche. Thus, we propose that the association of *Drosophila* with yeast species from new habitats can lead to niche specialization processes and may be a driving force behind new speciation events and the establishment of novel insect adaptations. We also found that larval and adult performance is differently affected by yeast species, perhaps caused by differences in nutritional needs in these two stages. Furthermore, we observed that the yeast preference of the female fly at oviposition sites does not necessarily correlate with optimized larval performance. Lastly, we provide evidence that *Drosophila* flies and yeasts from a shared habitat together participate in activities of niche construction, which

in return enhance both insect and yeast performance on a given substrate. Thus, we propose that both partners mutually benefit from specific associations of fly and yeast (Fig. 1C).

Material and methods

Fly stocks

For our study, we chose to work with the cosmopolitan *Drosophila* species *D. melanogaster* and two species with a specialized lifestyle, *D. mojavensis* and *D. putrida*, which are found on cacti and mushrooms, respectively. Experiments were performed with wild-type strain *Drosophila melanogaster* Canton-S (WTcs, stock no. 1), *Drosophila mojavensis* ssp. *mojavensis* (Cornell stock no. 15081-1352.10, Ithaca, NY, USA) and *Drosophila putrida* (Cornell stock no. 15150-2101.00, Ithaca, NY, USA). *Drosophila melanogaster* and *D. mojavensis* flies were maintained on standard diet (Supplementary material Appendix 2 Table A1). For the rearing of the mycophagous *Drosophila* species, *D. putrida* standard diet was supplemented with store-bought, organic, Portabella mushroom slices (*Agaricus bisporus* (Grimaldi 1985)). The *D. melanogaster* stock was kept at 25°C and 70% RH, with a photoperiod of 12 h:12 h Light:Dark (Stökl et al. 2010). *Drosophila mojavensis* and *D. putrida* flies were reared at room temperature with ambient light and humidity conditions (approximately 22°C and 16 h:8 h light:dark with 40% RH).

Host purée

Organic banana and Portabella mushrooms were purchased at a local organic grocery store while the cactus host material (*Opuntia phaecantha* v. *tenuispina*) was ordered from Uhlig Kakteen (Germany). These organic materials were homogenized with a hand-held blender, then transferred into 50 ml reaction tubes and kept at −20°C until further use.

Yeast maintenance

Yeast species were selected based on published association with the three target *Drosophila* species and their hosts (banana, cactus and mushroom). According to a literature search, the yeast species *Pichia cactophilia* and *Vanrija humicola* were assumed to be unique for their host association (cacti and mushrooms, respectively (Gilbert 1980, Grimaldi 1985)), while *Saccharomyces cerevisiae* is known to be rather ubiquitous, with a cosmopolitan distribution similar to its vector, *D. melanogaster* (Supplementary material Appendix 2 Table A2, Piskur et al. 2006, Rozpedowska et al. 2011). Yeast strains were purchased from the National Collection of Yeast Cultures (NCYC, Norwich, United Kingdom; *S. cerevisiae* NCYC 505, *P. cactophilia* NCYC 1492) and the Centraalbureau voor Schimmelcultures (CBS, Utrecht, Netherlands; *V. humicola* CBS 571). Aliquots of each yeast species were stored in 20% glycerol solutions at −80°C for long term storage. Fresh yeast cultures were maintained on yeast malt (YM) agar plates (yeast extract 3 g l^{-1}, malt extract 3 g l^{-1}, D-glucose 10 g l^{-1}, peptone 5 g l^{-1} and 2% agar at 25°C and 80% RH. Liquid cultures of all yeast species were freshly prepared every alternate day by inoculating 40 ml of YM medium (yeast extract 3 g l^{-1}, malt extract 3 g l^{-1}, D-glucose 10 g l^{-1}, peptone 5 g l^{-1}) with a single isolated colony and grown in 50 ml reaction tubes at 25°C with 250 rpm. The liquid yeast cultures served as pre-cultures for experiments and were grown until they reached stationary phase conditions with an OD_{600} of 2 (optical density at 600 nm). The pre-cultures were then spun down in a centrifuge at 320 g for 3 min, the supernatant was discarded, and the pellet was resuspended in 15 ml fresh, sterile YM medium.

Attraction assays

For each *Drosophila* species and fly host combination we selected one yeast species that has been isolated from the flies' host or has also been found in association with the respective *Drosophila* species (Supplementary material Appendix 2 Table A2). In trap assays, the ability of each of the three target *Drosophila* species was tested to differentiate between the target yeast species using their sense of olfaction (Fig. 2A). Furthermore, the trap assays provided information about the attraction of the target *Drosophila* species towards the different yeast species. For this, we simultaneously presented 40 flies of the same species at a 1:1 sex ratio with four options: traps filled with 2 ml of the yeast malt (YM) growth medium alone (control) or 2 ml YM inoculated with one of the three different yeast species grown to an OD_{600} of 2 and allowed each *Drosophila* species to display their preference (Fig. 2A). The plastic traps consisted of 20 ml containers (Specimen container, SAMCO6-0181, VWR, Darmstadt, Germany), which had a paper cone lid to keep the flies from leaving the container once they had entered it. In addition, 200 µl of mineral oil was pipetted onto the surface of all four treatments to help capture flies upon contact. Traps were placed at corners of a square with a side length of 10 cm and in the middle of a 50 × 50 cm mesh insect rearing cage. Assays were run in a climate chamber (dimensions 3 × 4.2 × 3.2 m, air exchange approximately 8600 $m^3 h^{-1}$) at 25°C, 70% relative humidity and a 12 h:12 h light–dark cycle. Flies were allowed to choose between the different traps for 24 h. After 24 h, the amount of flies outside of the traps was documented as well as the numbers of flies in the traps. Additionally, the sex of flies trapped was noted.

Oviposition assays

In the oviposition assays flies were simultaneously provided with three oviposition plates that only varied in the supplemented yeast species, allowing each *Drosophila* species to display any preference for oviposition (Fig. 2C). Prior to the oviposition assays, seven to eight-day old flies were separated into groups of 20 females each, which were then paired with

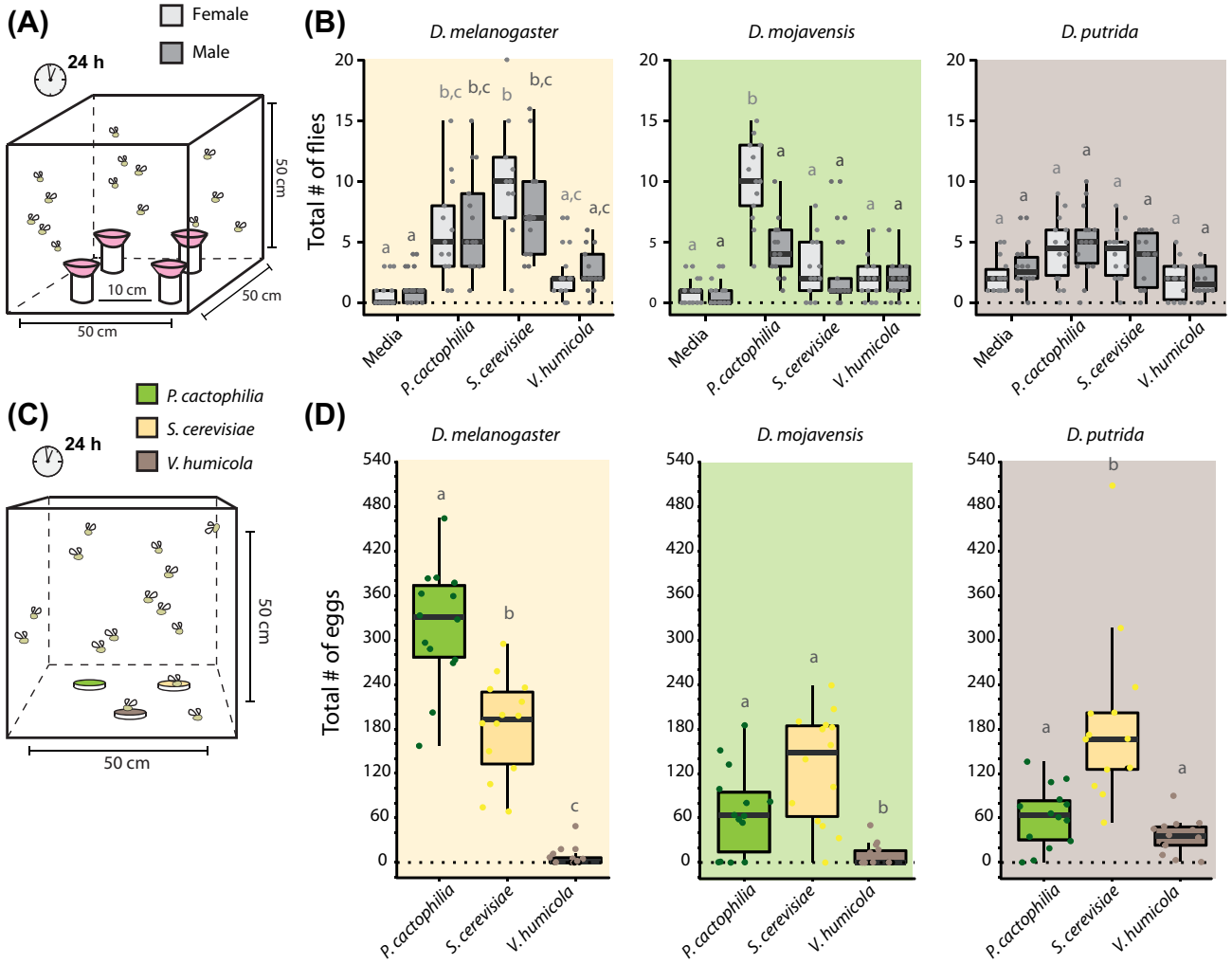

Figure 2. Behavioral preferences of *Drosophila* species towards different yeast species without the background of host material. (A) Trap assays in which the flies were simultaneously presented with three different yeast species, as well as the growth medium (YM) as a control. Colored cones served as trap entrances. (B) Total number of flies caught in the different traps of the attraction assay separated by sex (n = 14 replicates with each 40 individuals at a 1:1 sex ratio). Significant differences are indicated with letters above the box plots (Friedman test with Dunn's post hoc test). Boxplots represent the median (bold horizontal lines) with the interquartile range (whiskers). (C) Design of oviposition assays, where gravid females could choose between three different oviposition plates, which consisted of modified standard diet inoculated with one of the tested yeast species. (D) Total numbers of eggs found on each oviposition plate for the three target *Drosophila* species in presence of the different yeast species. (n = 14 replicates with each 20 female flies.) Letters above the box plots indicate significant differences between the numbers of eggs found on the oviposition plates (Friedman test followed by Dunn's multiple comparison post hoc test). Box plots show the median (bold horizontal lines) and whiskers the interquartile range.

five males per group to ensure a mated status of the females for the experiments. These flies were kept 24 h on food with standard diet that was supplemented with a yeast extract/peptone mixture (1:1) to guarantee the availability of gravid females and reliably high egg counts for these assays. The oviposition plates had a diameter of 60 mm and consisted of 7 ml standard diet, which lacked brewer's yeast and the antifungal growth component nipagin. Additionally, the plates were inoculated with 400 µl of stationary phase yeast pre-culture at an OD_{600} of 2 and incubated for 24 h at 25°C, all while flies were kept on the yeast extract/peptone mixture. For these assays, three oviposition plates, each inoculated with one of the target yeast, were placed in the middle of a 50×50 cm mesh insect rearing cage and spaced 10 cm apart (Fig. 2C). Females were allowed to oviposit for 24 h. Assays were kept under controlled temperature and humidity conditions (25°C and 70% RH with a light–dark cycle of 12 h:12 h). For all three *Drosophila* species, 14 replicates were conducted. After 24 h, the oviposition plates were removed from the mesh cages and immediately imaged for subsequent egg counts. Images of the plates were taken with an Axio Zoom V16 at a magnification of 12.5 (PlanApo Z 0.5× objective, 0.125 NA). For this, the tile scan function of the Zeiss software (ZEN 2 Blue edition, ver. 2.0.0.0) was applied. The magnification of 12.5 led to a total amount of 28

tiles per imaged oviposition plate, which were each processed into a final image using the software's built-in stitching method with a tile overlap of 10%. The resulting images were analyzed in FIJI (ImageJ ver. 1.51 k; NIH) utilizing the 'multi-point' option, which marked every counted egg with a symbol and helped avoiding miscounts.

Standard diet supplemented with host purée and inoculated with yeast

In these assays flies were simultaneously presented with all three yeast species across a single host material (banana, cactus or mushroom purée, Fig. 3A–B). Flies were prepared as described for the oviposition assays with standard diet inoculated with yeast. The base of the oviposition plate consisted of modified artificial diet (no brewer's yeast and no nipagin). The oviposition plates were first inoculated with 400 µl of stationary phase yeast pre-culture ($OD_{600} = 2$). Then we covered approximately half of the diet evenly with 2 g of homogenized host material. Finally, the half of the plate with host purée was inoculated with 200 µl of additional pre-culture of one of the three target yeast species. Plates were incubated for 24 h at 25°C. By only partially covering the surface of the oviposition plates with the host purée it was possible to observe potential preferences of the flies towards either modified standard diet inoculated with yeast alone, or towards host purée in the presence of yeast culture. Assays were set up and run as mentioned above (Fig. 3A). Likewise, oviposition plates were processed as explained previously, with the addition that for each plate two final images from two focus planes were compiled. These two imaging heights were necessary, as the extra

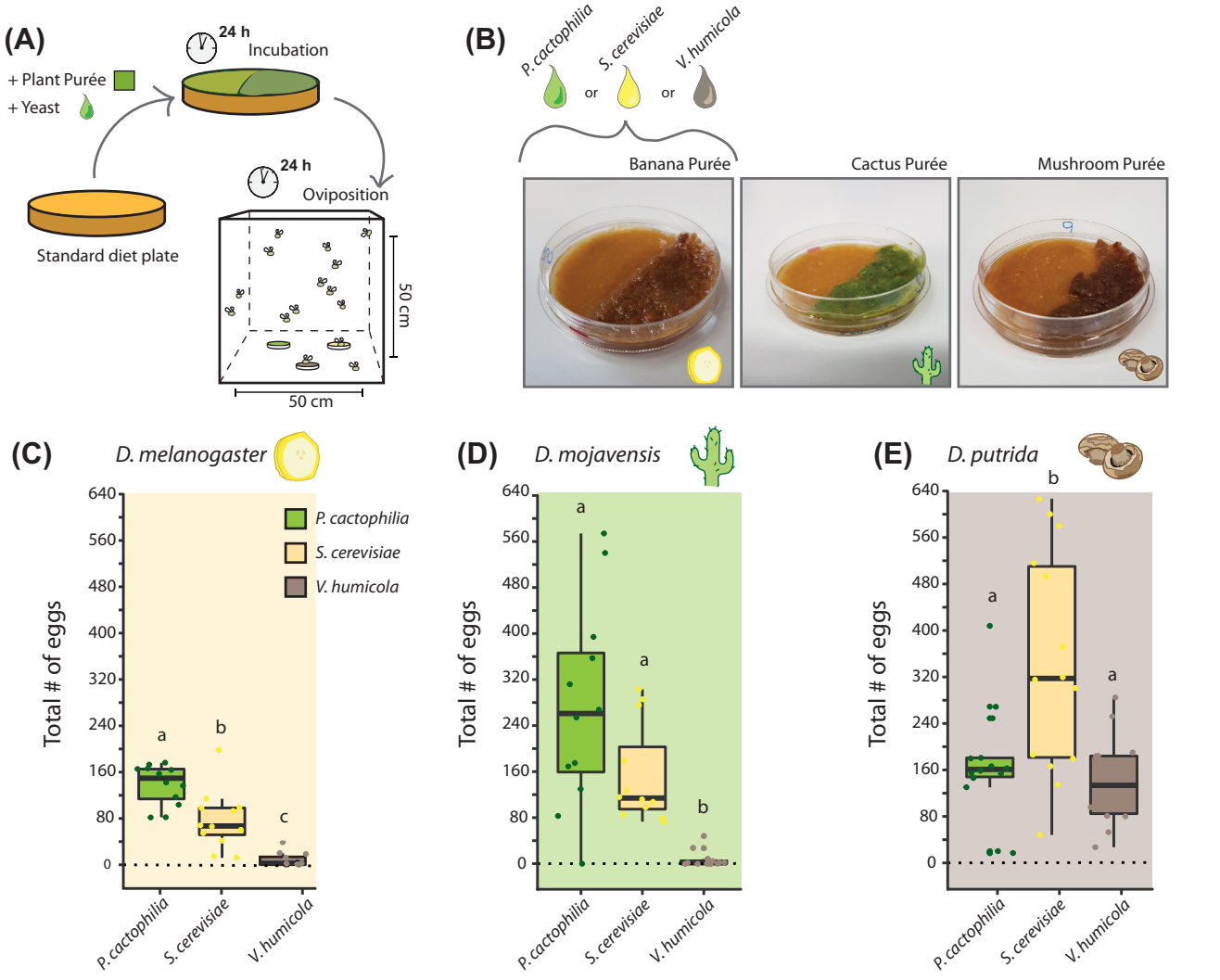

Figure 3. *Drosophila* oviposition preference on host material inoculated with different yeast species. (A) Oviposition assays on host material inoculated with target yeast species. (B) Examples of oviposition plates with the three different host materials. (C–E) Total number of eggs laid by females of *D. melanogaster* (C), *D. mojavensis* (D) and *D. putrida* (E) on the different host material. Letters above the box plots indicate significant differences between the numbers of eggs found on the oviposition plates ((C–D) Friedman test followed by Dunn's multiple comparison post hoc test, (E) one-way ANOVA with Tukey–Kramer multiple comparison post hoc test; n = 12–14 replicates with each 20 female flies). Boxplots represent the median (bold horizontal lines) with the interquartile range (whiskers).

layer of host purée on top of the modified diet did not allow capturing all eggs in focus using just one image.

Larval development assays

Larval development was evaluated as the amount of days needed for reaching two milestones in a fly's life, (A) pupation and (B) adult eclosion, on modified standard diet and in the presence of the different target yeast species. Prior to the assays, small plastic vials (28.5×95 mm) were prepared by adding 5 ml of modified standard diet to the vials, which lacked brewer's yeast and the antifungal substance nipagin. Under a sterile bench, the surface of the modified standard diet was broken up for an easier access of yeast culture and larvae, then 200 µl of yeast pre-culture at an OD_{600} of 2 were added to the diet. For each of the three target yeast species, 15 replicates were set up and incubated for 24 h at 25°C. From oviposition plates a total of 15 first instar larvae of one *Drosophila* species were transferred per rearing vial. The oviposition plates consisted of 2% agar and had the addition of some broken up pieces of modified standard diet supplemented with a yeast extract/peptone mixture (1:1) in the center of the plate. The yeast extract/peptone mixture served as an oviposition stimulus for the adults. After the addition of first instar larvae, vials were checked twice per day for the appearance of pupae, and after pupation for the eclosion of adults until zero new flies emerged for two consecutive days.

Fly phenotyping

The influence of dietary yeast and other yeast-dependent effects on the performance of the different *Drosophila* species was measured in regards to ovary size as a proxy for fecundity (Boulétreau-Merle et al. 1982, R'kha et al. 1997, Klepsatel et al. 2013, Mendes and Mirth 2016), and the weight of adult female flies was used as an assessment of overall adult fitness. Flies for the measurements originated from the larval development assays and were taken from the vials after eclosion for the determination of adult weight and for the dissection of the developed ovaries in *D. melanogaster*, *D. mojavensis* or *D. putrida* respectively.

Adult weight

Weighing of flies preceded ovary dissection but happened in succession to enable statements about a possible correlation between adult weight and their ovary size. For every treatment, 25 females randomly selected from the different replicates were measured where available; otherwise, all females that hatched were weighed. In the case of *D. melanogaster* female flies were measured two days after eclosion, and in the case of *D. mojavensis* and *D. putrida*, six days after eclosion. The two *Drosophila* species *D. mojavensis* and *D. putrida* (Koerte et al. unpubl.) mature slower than *D. melanogaster* (Carracedo et al. 1989) and their ovaries would have been underdeveloped independent of dietary reasons if dissected too early. Flies were removed from vials of the larval development assays and sorted on a CO_2 pad by their respective sex. Female flies from the same species were pooled together by treatment and cooled on ice in 1.5 ml reaction tubes. After 30 min on ice individual flies were weighed on an analytical scale.

Ovary size

Subsequently to the weight measurements, ovaries of these females were dissected. For each treatment and each species, ovaries of 15 females were dissected. The flies were cooled on ice and dissected in 1 M phosphate-buffered-saline (PBS) with 0.1% Triton X 100. Images of the ovaries were taken with an Axio Zoom V16. All ovaries were scanned at a magnification of 50 (PlanApo Z 0.5× objective, 0.125 numerical aperture (NA)). The ovary size was measured in FIJI (ImageJ ver. 1.51 k; NIH) by tracing the outline of individual ovaries and calculating the size of the regions of interest after predefining the pixel by pixel size in the settings according to the scan information from the original image taken in the Zeiss software (ZEN 2 Blue edition, ver. 2.0.0.0).

Gas chromatography–mass spectrometry (GC–MS) headspace analysis

After confirming that the tested *Drosophila* species were able to distinguish between the target yeast species based on olfactory cues (Fig. 2B), the odor profile of target yeast species and YM medium was characterized to quantitatively compare relative abundance of odorants and to possibly identify species-specific yeast odorants. For this, headspace of 5 ml YM medium or yeast culture at an OD_{600} of 2 was collected for 30 min with a solid-phase microinjection (SPME) fiber. After manual injection, samples were analyzed via a GC–MS interfaced to an inert XL MSD unit device with an installed HP-5MS UI column (19091S-413U, Agilent Technologies). For each GC–MS run, the temperature of the oven was initially held at 40°C for 3 min, and then increased in increments of 20°C min^{-1} up to 260°C. Mass spectra were recorded in scan mode from 33 to 350 m/z with electron impact (EI) ionization at 70 eV and 300 mA. Resulting GC–MS profiles were characterized by matching the ion profiles of identified odorants to a standard library (NIST Mass spectrum library) using MSD ChemStation (F.01.02.2357, Agilent). For all three yeast species as well as the growth medium, three replicates were produced for verification.

Electroantennogram (EAG) recordings

In electroantennograms (EAGs, Supplementary material Appendix 1 Fig. A4A), the summed activity of sensory neurons on the antenna of target *Drosophila* species was recorded in response to each yeast pre-culture, the YM medium and the host purée. Recordings were used to ascertain within each yeast species whether the flies perceived the different odor profiles in a discriminative manner. For these recordings, flies of seven to nine days of age were immobilized in truncated 200 µl pipette tips. The Ringer solution filled Ag–AgCl glass

capillary reference electrode was placed into the fly's compound eye and of the recording electrode (filled with the same solution) was placed against the antenna's third segment (Supplementary material Appendix 1 Fig. A4A), which all took place under a microscope (10× magnification, 0.30 numerical aperture [NA]) and with the help of a micromanipulator. Ringer solution consisted of 1.0 mM $CaCl_2$, 22.5 mM glucose, 1.5 mM HCl, 171.9 mM KCl, 9.2 mM KH_2PO_4, 10.8 mM K_2HPO_4, 3.0 mM $MgCl_2 \cdot 6H_2O$, 12 mM NaCl at a pH of 6.5 and an osmolarity of 475 mOsmol l^{-1}. The odor stimulus elicited antennal response was generated using a 10× pre-amplified signal headstage, converted via an amplifier and then recorded, visualized and analyzed via Syntech Autospike ver. 3.7. For the odor stimulus, 10 µl of yeast culture (OD_{600} = 2) or YM medium or 30 mg of host purée was used. For each stimulus, seven to eight biological replicates were recorded per *Drosophila* species.

Observations of host decay

Prior to the assays, 30 *Drosophila* males per replicate were allowed to feed on 50 µl of pre-culture (OD_{600} = 2) from one of our selected yeast species, respectively. Host material was added on top of standard artificial fly diet. At the beginning of the assays, male flies were transferred into vials containing samples of the host material. *Drosophila melanogaster* males were kept on banana slices, *D. mojavensis* males on pieces of cactus, and *D. putrida* males were transferred to vials with mushroom slices. As a control, vials were prepared that only contained standard diet and host material, and not flies. The course of decomposition of the host material was documented for ten days, with pictures being taken on day 1, 3, 7 and 10 (Supplementary material Appendix 1 Fig. A6).

Sugar content measurements

In order to indirectly asses yeast fitness and performance on the respective *Drosophila* host material, the capability of the target yeast species to induce or even accelerate fermentation progress was assessed by measuring sugar contents of host purée inoculated with each yeast species or the growth medium (Supplementary material Appendix 1 Fig. A5A). For these measurements, 2.0 g of purée were weighed and transferred into small, sterile petri dishes (35 mm). The purée was then inoculated with 250 µl of yeast pre-culture at OD_{600} of 2 or supplemented with 250 µl YM medium. For each yeast species and the control (YM medium), three replicates per host purée were prepared. The purée was well mixed and incubated at 25°C and 80% RH for 72 h. After incubation, the purée was mixed again, and 100 mg of material was removed and transferred into 1.5 ml polypropylene reaction tubes. Subsequently, 1 ml of 70% methanol was added to the host material and samples were thoroughly vortexed for 2 min. Next host material was centrifuged at 16 000 rcf for 5 min to separate the extract from the solid phase. The supernatant was carefully removed, without disruption of the solid phase, and then transferred into 1.5 ml reaction tubes. Then, the supernatant was concentrated under N_2 at 35°C. Samples were resuspended in 100 µl of water and diluted 1:5000 before injection. Sugars were analyzed on an HP 1200 series coupled to an API 5000 triple-quadrupole mass spectrometer with an apHera NH_2 Polymer column (150 × 4.6 mm, 5 µm). A chromatographic gradient of water (solvent A) and acetonitrile (solvent B) with a flow rate of 1 ml min^{-1} at 20°C was used, and the following program was run: 80% B (0.5 min), 80–55% B (12.5 min), 55–80% B (1 min), hold at 80% for 4 min. The mass spectrometer was operated in the negative mode with collision-activated decomposition, using a curtain gas pressure of 35 psi, with a collision gas pressure of 70 psi, an ion spray voltage of −4500 eV and a turbogas temperature of 700°C. Compounds were detected using the scheduled multiple reaction monitoring (MRM) outlined in Supplementary material Appendix 2 Table A3. The MRM window was 300.0 s, the target scan time was 1.00 s and the cycle time was 1.00 s, with 1081 cycles. Analyst ver. 1.5 software was used for data acquisition and processing.

Figure design and statistics

All figures were generated in RStudio ver. 1.2.1335 (RStudio Team 2018) and then adjusted for layout and graphical design in Adobe Illustrator CS5 ver. 15.0.0. Statistics were performed in GraphPad InStat ver. 3.10 and the outcome of statistical analysis was added to the pre-processed figures. For all choice-experiments (attraction trap assays and oviposition assays with and without host purée, Fig. 2B, D, 3C–E) the results for the different choices (target yeast species or control) were matched, but not all parametric. Consequently, the data was analyzed via the repeated measures Friedman test, followed by a Dunn's multiple comparison post hoc test. The results of the larval development experiments (Fig. 4) were analyzed in a comparison of larvae or fly numbers at the end of the recording window. As all treatments (different dietary yeast species) were independent of each other, the data was either tested for significant differences based on a one-way ANOVA if parametric (Fig. 4B, *D. melanogaster*) followed by a Tukey Kramer post hoc test or if non-parametric using the Kruskal–Wallis test followed by a Dunn's multiple comparison post hoc test (Fig. 4A–B, *D. mojavensis*, *D. putrida*). The same rationale was used for the statistical analyses of the data on ovary size and adult weight of the females originating from the larval development assays (Fig. 5B, ovary size *D. melanogaster*: Kruskal–Wallis test with a Dunn's post hoc test, *D. mojavensis*: one-way ANOVA followed by a Tukey Kramer post hoc test; adult weight *D. melanogaster* and *D. mojavensis*: one-way ANOVA followed by a Tukey Kramer post hoc test). The preference index of the different *Drosophila* species for the two different sites of the oviposition plates in choice assays with host purée (Supplementary material Appendix 1 Fig. A1A) was statistically substantiated by testing for a significant difference from zero using a two tailed one-sample t-test as the data was normally distributed. The individual p-values are provided in Supplementary material Appendix 1 Fig. A1B–D. For the analyses of the amounts of eggs laid in oviposition assays with

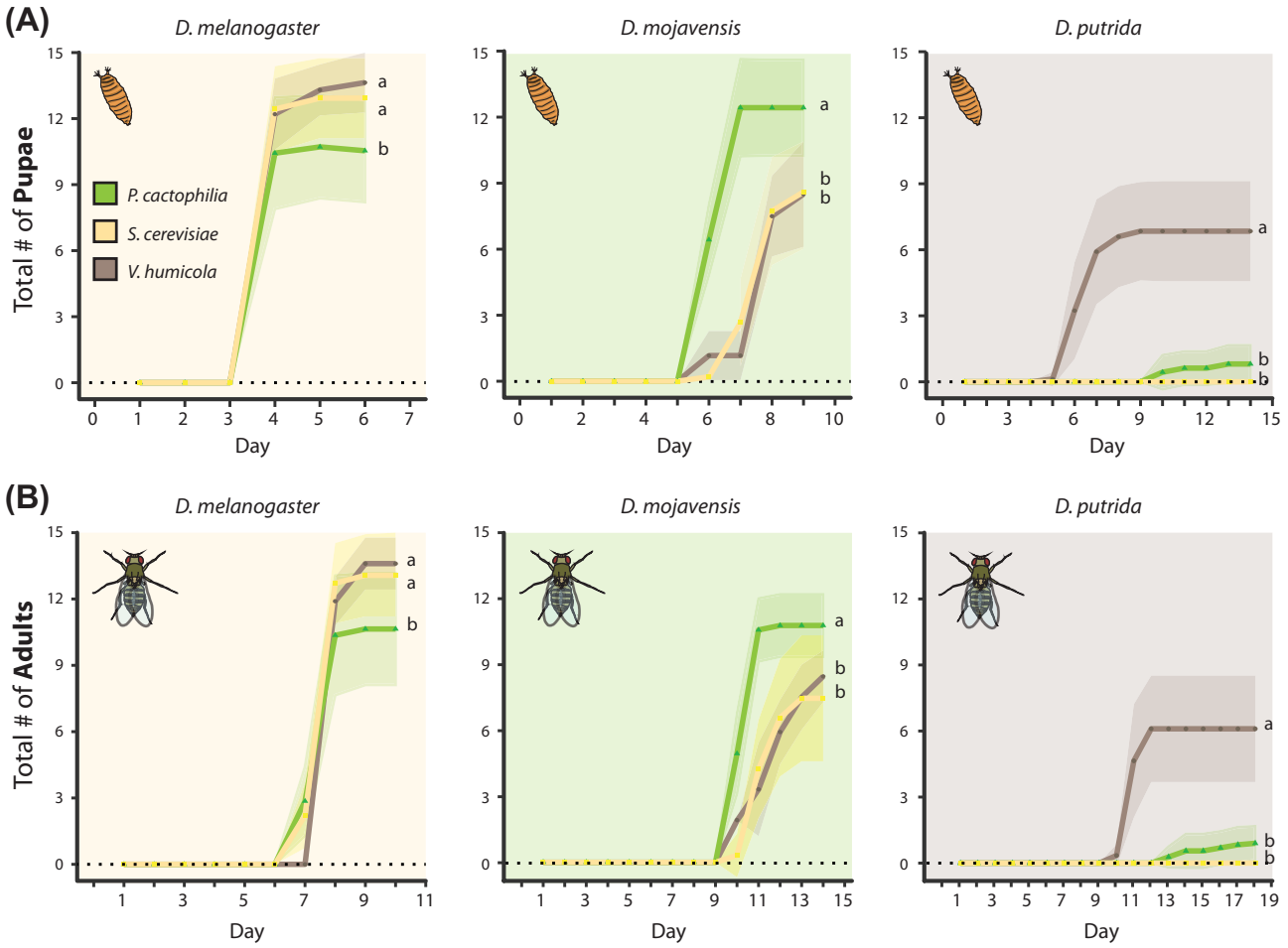

Figure 4. Effects of target yeast species on larval developmental time metrics and survivorship. (A) Average numbers of pupae (n = 15 replicates with each 15 first instar larvae) that were counted during each day in vials with different yeast species. Ribbons around line plots represent the standard deviation for the pupae number per day. (B) Average numbers of adults emerging from pupae in vials with different yeast species. Ribbons show the standard deviation from the average per day. Letters at the end of the line plots indicate significant differences between the different yeast treatments based on data from the last day of the recording window ((B, *D. melanogaster*) one-way ANOVA with Tukey–Kramer multiple comparison post hoc test; all other data in (A–B) were analyzed via a Kruskal–Wallis test with a Dunn's multiple comparison post hoc test).

and without host material (Supplementary material Appendix 1 Fig. A2), we compared the distribution of the two data groups per *Drosophila* species using an unpaired t-test including a Welch correction if normally distributed (Supplementary material Appendix 1 Fig. A2, *D. putrida*) or if non-parametric with a two-tailed Mann–Whitney test (Supplementary material Appendix 1 Fig. A2, *D. melanogaster*, *D. mojavensis*). The p-values can be found in Supplementary material Appendix 1 Fig. A2. Data from the EAG recordings (Supplementary material Appendix 1 Fig. A4B–D) with different odor stimuli that were measured in succession was matched and thus analyzed for statistical significance if parametric with a repeated measures ANOVA followed by Tukey–Kramer multiple comparison post hoc test (Supplementary material Appendix 1 Fig. A4B) and if non-parametric with a Friedman test and a subsequent Dunn's post hoc test (Supplementary material Appendix 1 Fig. A4C–D).

Results

Attraction of flies towards yeast cultures

Our first interest was to examine whether our target *Drosophila* species, *D. melanogaster*, *D. mojavensis* and *D. putrida*, can distinguish between different yeast cultures and whether they show species-specific preferences in their attraction towards the provided yeast (Fig. 2A–B). For *D. melanogaster* adults, we observed an attraction towards *P. cactophilia* as well as *S. cerevisiae* cultures. Traps with YM medium contained significantly less flies (Fig. 2B).

In the case of *D. mojavensis* females, we observed a strong preference in attraction for *P. cactophilia* while none of the other yeast species produced significant attraction different from the control. Although little information is available about the yeast associated with *D. putrida*, this fly species is

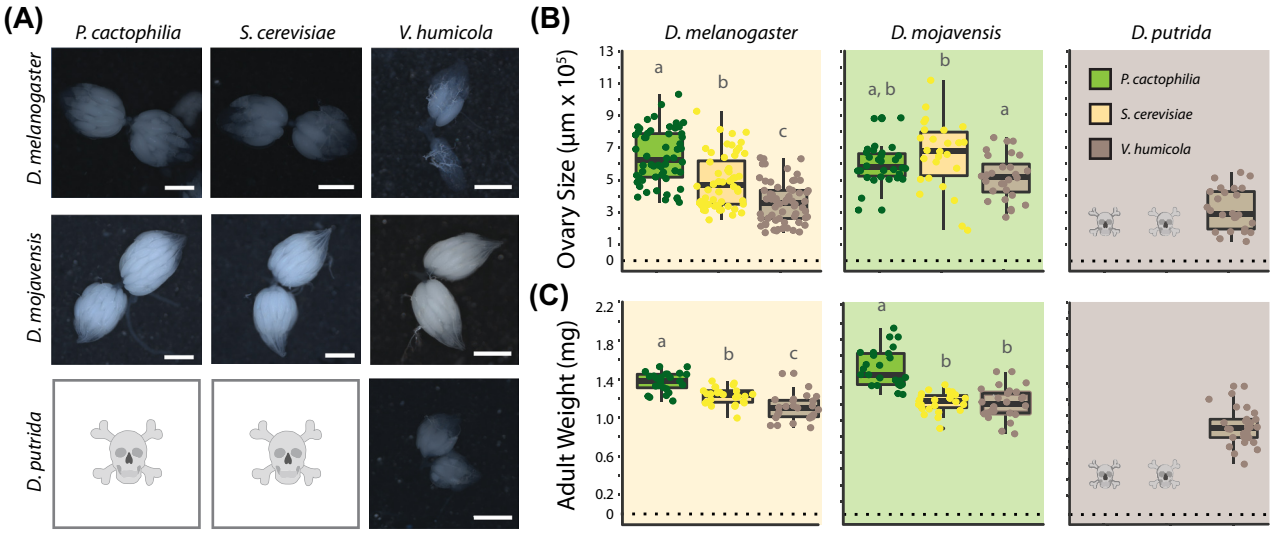

Figure 5. Ovary size and adult weight of flies reared in association with different yeast species. (A) Representative images of dissected ovaries from adult females of all three *Drosophila* species raised on an artificial diet supplemented with one of the indicated yeast species (scale bar 500 µm). Scull symbols indicate treatments in which no larvae survived, or no females hatched. (B) Measurements from ovary surface area of female flies (n = 25–30 ovaries per species and yeast treatment). Letters above the box plots indicate significant differences in ovary size between the yeast species treatments (*D. melanogaster*: Kruskal–Wallis test followed by Dunn's multiple comparison post hoc test, *D. mojavensis*: one-way ANOVA with Tukey–Kramer multiple comparison post hoc test). Box plots show the median (bold horizontal lines) and whiskers the interquartile range. (C) Weight of females used for the ovary dissection in (B). Letters above the box plots indicate significant differences (one-way ANOVA followed by Tukey–Kramer multiple comparison post hoc test). For each data set the median is shown in a bold horizontal line and the interquartile range is represented by the whiskers of the box plots. Scull symbols were used if all larvae died before eclosion or if adults which emerged had been males only.

known to be mycophagous (Grimaldi 1985). In our attraction assays with *D. putrida* we did not find significant differences in preference towards our target yeast species or the growth medium when we differentiated between fly sex. However, if we pooled the data, total fly numbers of *D. putrida* caught in traps with *P. cactophilia* culture were significantly higher than in traps with the other two yeast species or the YM control.

Oviposition assays with yeast on artificial diet

After ascertaining that the target *Drosophila* species can differentiate between selected yeast species and that the flies show species-specific attraction, we next pursued choice-tests of egg-laying behaviors using artificial diet containing the three yeast species (Fig. 2C–D). *Drosophila melanogaster* females showed a preference to oviposit on plates inoculated with *P. cactophilia* or *S. cerevisiae*, preferring *P. cactophilia* over *S. cerevisiae*. Plates with either of these yeast species were significantly different compared to *V. humicola* plates, where few if any eggs were deposited (Fig. 2D). In oviposition assays with *D. mojavensis* we found again a preference for both *P. cactophilia* as well as *S. cerevisiae*, in this case with a trend for oviposition in presence of *S. cerevisiae*. Similarly, *D. putrida* also preferred oviposition plates inoculated with *S. cerevisiae*. We observed that *D. putrida* laid a higher average number of eggs on plates containing *V. humicola* than either of the other two *Drosophila* species (Fig. 2D, *D. melanogaster* 7 ± 13, *D. mojavensis* 7 ± 15, *D. putrida* 35 ± 23). Thus, despite its preference for *S. cerevesiae*, *D. putrida* (Fig. 2D, *D. putrida*, Supplementary material Appendix 1 Table A4) appeared to be more willing to accept mycophagous yeast and to lay eggs in the presence of a yeast species that was seemingly less desirable for the other two *Drosophila* species.

Oviposition assays with yeast and host material

In order to examine the role that host substrate and yeast play in *Drosophila* oviposition preference, we next performed oviposition assays using host material in addition to our target yeast species (Fig. 3). Here, the flies were simultaneously presented with all three yeast species across a single host material in the same cage (Fig. 3A–B). For *D. melanogaster*, we observed a nearly identical trend to that observed when we presented the yeast alone without any host material (Fig. 3C). Moreover, the preference for *P. cactophilia* was even more pronounced when combined with banana purée, though we did not observe changes in the oviposition preference of *D. melanogaster* females towards *S. cerevisiae* or *V. humicola* in the presence of the host material (Fig. 3C). Again, there were only a few eggs laid on plates containing *V. humicola*, even in combination with banana purée. When we had a closer look at which half of the oviposition plates the *D. melanogaster* females favored, we found a clear preference of the females for the half containing banana purée (Supplementary material Appendix 1 Fig. A1, one-sample t-test: *P. cactophilia* $p < 0.0001$, *S. cerevisiae* $p < 0.0001$, *V. humicola* $p = 0.0176$).

For *D. mojavensis*, we observed that when host and yeast were combined, females now showed a tendency to lay eggs preferably on the substrate inoculated with *P. cactophilia* (Fig. 3D). Interestingly, we also found a drastic increase in total egg numbers laid by *D. mojavensis* in these trials when we combined yeast with their natural host material (Supplementary material Appendix 1 Fig. A2, two-tailed Mann–Whitney test: $p = 0.0005$), especially as compared to the trials with yeast alone; however, we again documented that hardly any oviposition occurred on plates inoculated with *V. humicola*. Females of *D. mojavensis* did not demonstrate a preference whether to lay eggs on the host material or the artificial diet sides of the plates (Supplementary material Appendix 1 Fig. A1, one-sample t-test: *P. cactophilia* $p = 0.6350$, *S. cerevisiae* $p = 0.6669$, *V. humicola* $p = 0.8495$).

In regard to *D. putrida*, this fly species significantly preferred to oviposit on *S. cerevisiae* but again laid also many more eggs than the other two *Drosophila* species on *V. humicola* (Fig. 3E, Supplementary material Appendix 1 Table A4). Additionally, on oviposition plates inoculated with *S. cerevisiae*, *D. putrida* favored the host material side of the plate (mushroom) as a substrate for oviposition (Supplementary material Appendix 1 Fig. A1, one-sample t-test: *P. cactophilia* $p = 0.4827$, *S. cerevisiae* $p = 0.0025$, *V. humicola* $p = 0.2438$). Similar to *D. mojavensis*, overall oviposition numbers were greatly increased in *D. putrida* when both yeast and host material (e.g. mushroom purée) were combined (Supplementary material Appendix 1 Fig. A2, unpaired t-test with Welch correction: $p < 0.0001$).

Larval performance in the presence of different yeast species

As we had now demonstrated adult preferences for both attraction and oviposition, we next wanted to examine whether there was a correlation of this adult preference in regards to subsequent larval development (Fig. 4). For all target *Drosophila* species, we monitored larval development, survivorship and the time point of adult eclosion on modified standard diet, which was mixed with one of the three yeast species. On average, *D. melanogaster* pupae first appeared on day four, with larvae performing best on a *S. cerevisiae* and *V. humciola* diet (Fig. 4A, *D. melanogaster*, Kruskal–Wallis test with Dunn's post hoc test). Larvae of *D. mojavensis* took longer than *D. melanogaster* larvae to reach pupation, with an average of six or seven days for most replicates. We noted that *D. mojavensis* larvae growing up on *P. cactophilia* developed on average one to two days faster than those which had been reared on the other two yeast species. Moreover, we also documented that more *D. mojavensis* larvae reached pupation when reared on diet supplemented with *P. cactophilia* (Fig. 4A, *D. mojavensis*, Kruskal–Wallis test with Dunn's post hoc test). In assays with *D. putrida* differences in larval development and survivorship were more drastic where few if any larvae survived when reared on artificial diet supplemented with *S. cerevisiae* or *P. cactophilia* (Fig. 4A, *D. putrida*, Kruskal–Wallis test with Dunn's post hoc test). Strikingly, only in the presence of *V. humicola* did we observe considerable numbers of *D. putrida* larvae to survive until pupation.

Similar to our examination of larval developmental rate, we also tracked adult eclosion from the pupal case for each *Drosophila* species in association with different inoculated yeast species (Fig. 4B). Here overall numbers of *D. melanogaster* adults that hatched were highest on diet containing *S. cerevisiae* or *V. humicola* (Fig. 4B, *D. melanogaster*, one-way ANOVA with a Tukey–Kramer post hoc test). However, adult eclosion was on average one day delayed when *D. melanogaster* larvae developed on artificial diet containing *V. humicola*. In assays with *D. mojavensis*, adults hatched fastest on average upon the yeast from their natural habitat, *P. cactophilia* (Fig. 4B, *D. mojavensis*, Kruskal–Wallis test with Dunn's post hoc test). If larvae had been reared in association with yeast that are less prevalent in their ecological niche, *S. cerevisiae* or *V. humicola*, we observed fewer overall adult *D. mojavensis* surviving until eclosion (Fig. 4B, *D. mojavensis*, Kruskal–Wallis test with Dunn's post hoc test). In the case of *D. putrida* the few larvae which made it until pupation in the presence of *P. cactophilia* also successfully eclosed, while larvae on a diet containing *S. cerevisiae* never reached further developmental stages beyond first or second larval instar. Only food supplemented with *V. humicola* led to *D. putrida* adult survivorship numbers higher than 6%, where survivorship was on average 39% in the presence of this mycophagous yeast species. Our data suggest that while the generalist, *D. melanogaster*, can perform equally well on a wide range of different yeast species, that both of the other *Drosophila* specialists display larger variation in developmental time as well as survivorship when ingesting and exposed to different yeast species. This may be related to evolutionary restrictions on larval development due to specific nutritional needs, and relate to host specialization of the latter two *Drosophila* species.

Adult phenotypes from individuals grown on different yeasts

We further analyzed the ramifications of insect lifecycles in association with the target yeast species by addressing adult fly performance and reproductive development (Fig. 5). Here, we analyzed these changes by collecting data on ovary size and adult weight as indicators for fecundity and overall fitness after being reared on different yeasts. In agreement with the adult preference for *P. cactophilia* (Fig. 2D, 3C), ovary size and adult weight of *D. melanogaster* females grown on diets containing *P. cactophilia* were both significantly increased compared to these metrics from females reared on the other two yeast species (Fig. 5B, *D. melanogaster*, Kruskal–Wallis test with Dunn's post hoc test). Development of *D. melanogaster* females was poorest in association with *V. humicola*, where ovaries generally appeared underdeveloped (Fig. 5A). In assays with *D. mojavensis* females, ovaries were comparable in size on all yeast treatments (Fig. 5B, *D. mojavensis*, one-way ANOVA with a Tukey–Kramer post hoc test); Highest weight measurements were documented for *D. mojavensis* females that emerged from larvae fed on diets

supplemented with *P. cactophilia* (Fig. 5B). Overall, *D. mojavensis* females preferred to lay eggs on diet inoculated with *S. cerevisae* (artificial diet plus yeast) or *P. cactophilia* (host material plus yeast), which are the yeast species that produced progeny with big, ovariole-rich ovaries and heavy, well-nourished females (Fig. 2D, 3D, 5). As larvae of *D. putrida* did not survive on diet containing *S. cerevisiae*, and as the few adults emerging from diet inoculated with *P. cactophilia* were all males, we could only assess measurements from females reared on *V. humicola* (Fig. 5).

Odor profile of target yeast species

After finding evidence that our target *Drosophila* species had a species-specific preference towards the different yeast species and thus were able to differentiate between these yeast species based on volatile odors, we next wanted to analyze the headspace of the three yeast species to compare their odor profiles and possibly identify any yeast species-specific odorants (Supplementary material Appendix 1 Fig. A3). From the three tested yeast species, *P. cactophilia* produced odorants in the highest abundance and variety while *V. humicola* emitted volatiles in the lowest abundance and diversity (Supplementary material Appendix 1 Fig. A3B–D). In general, *V. humicola* was also the only yeast species in whose headspace we did not find evidence for the presence of 2-phenyl-1-ethanol. This odorant is known to be highly attractive for several *Drosophila* species and is associated with fermentation processes (Supplementary material Appendix 1 Fig. A3D, Becher et al. 2012, 2018). For the vinegar fly *D. melanogaster*, beside 2-phenyl-1-ethanol, eight additional volatile compounds produced by yeasts have been identified to induce upwind attraction leading to landing behavior at the source (Becher et al. 2018). In our analysis of the volatiles emitted by *S. cerevisiae* we found four out of these nine compounds (Supplementary material Appendix 1 Fig. A3C), while the headspace of *P. cactophilia* contained six odorants described as attractive to flies and *V. humicola* only produced two out of these nine volatiles. Most of the additional odorants identified solely in *P. cactophilia* samples were esters that are naturally also found in fruity bouquets such as ethyl propionate, isobutyl acetate and ethyl butyrate (Supplementary material Appendix 1 Fig. A3B).

The three *Drosophila* species showed antennal responses in electroantennogram (EAG) recordings towards each yeast species indicating that all tested *Drosophila* species were capable of detecting the odor bouquets emitted by each of the different yeast samples (Supplementary material Appendix 1 Fig. A4B–D). However, whether the antennal sensitivity of the three species significantly differs towards the individual yeast headspaces and whether this corresponds to observed *Drosophila* species-specific differences in their respective yeast preference remains to be determined in future work.

Effects of yeast presence on host decay

After we had studied the attraction, oviposition, larval development and progeny fitness of our three *Drosophila* species towards selected yeast species, we next aimed to learn if and how our target yeast species affected host decay. Here, we wanted to draw conclusions about a possible release and provision of nutritional factors for *Drosophila* adults and larvae via host decomposition through the presence of the associated yeast species. Furthermore, we examined indicators for differences in the yeast performance itself on the three different *Drosophila* hosts.

First, we wanted to assess if the three target yeast species are capable of differentially accelerating fermentation processes across our host material. We expected that changes in sugar contents, especially of the monosaccharides, in the host purée could be used as an approximate indicator for fermentation processes, yeast performance and propagation on the respective host material.

We analyzed sugar contents (fructose, glucose, an unknown monosaccharide, sucrose and two unknown disaccharides) of host purée, which had been inoculated with one of the three tested yeast species. Performance levels and metabolic activity of our three tested yeasts differed depending on the available host material. In the case of banana purée and *S. cerevisiae*, where we know most about yeast metabolism, host association and host composition, our data indicated that a yeast species linked to a specific habitat induced fermentation processes and converted available sugars at a faster rate than yeasts from other ecological niches (Supplementary material Appendix 1 Fig. A5B). However, for cactus and mushroom purée results were not conclusive enough to allow for informative statements about yeast performance and propagation (Supplementary material Appendix 1 Fig. A5C–D).

Next, for each host, we qualitatively analyzed the rate of decay after exposure to males of each *Drosophila* species, where flies were allowed to feed on one of the three selected yeast species for 24 h prior to the assays. We observed the formation of an opaque biofilm on host material and surrounding artificial diet in vials containing males that had fed on our different yeast species (Supplementary material Appendix 1 Fig. A6). In control vials with host material and without flies, no such biofilm was visible; instead, the control host samples were often rapidly overgrown with mold. In the presence of *Drosophila* males and a visible biofilm, the host material appeared to be broken down faster than the control samples and showed fewer signs of desiccation (Supplementary material Appendix 1 Fig. A6).

Discussion

Pioneering research as well as recent studies on the interaction of *Drosophila* species and their naturally associated yeast species have started to uncover the general principles that underlie interactions between these fly species and two other trophic levels; namely, their yeast communities and their host substrate (Cooper 1960, Becher and Guerin 2009, Anagnostou et al. 2010a, Becher et al. 2012, Stamps et al. 2012, Buser et al. 2014, Bellutti et al. 2018, Grangeteau et al. 2018, Murgier et al. 2019).

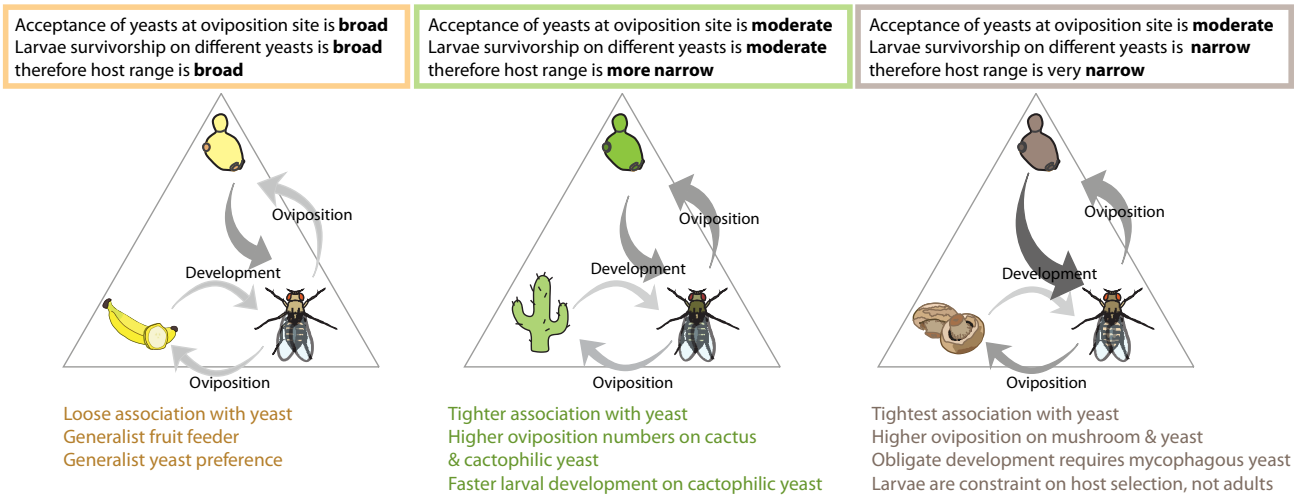

Figure 6. Model of *Drosophila*–host–yeast interactions. Schematic drawing describing the dependency of target *Drosophila* species on the association with specific yeast communities and the presence of certain host material for their oviposition preference and larval performance.

In our experiments, we found that the host range of tested *Drosophila* species correlates with the acceptability of different yeast species in food resources and at oviposition sites (Fig. 6). The generalist *D. melanogaster* can populate a broad range of host substrates. We found that this fly species accepts a variety of yeast species from different groups at breeding sites and the larval performance is consistently high in the presence of diverse yeasts. However, the specialists, *D. mojavensis* and *D. putrida*, either favor yeast species from their natural environments for oviposition or their larval survivorship is negatively affected by yeasts from unfamiliar habitats, keeping their host range narrow (Fig. 6). Thus, we propose that the larval development of more specialized *Drosophila* species is more sensitive to changes in associated yeast communities than that of generalist *Drosophila*.

On the basis of previous work (Scheidler et al. 2015), we initially predicted that *Drosophila* species from different ecological niches would be most attracted to and prefer yeast species associated with their respective habitat or host. Our reasoning behind this assumption was that yeasts would want to ensure transfer to suitable habitats by producing a specific composition of chemosignals especially attractive to *Drosophila* species linked to their ecological niche and in exchange, the flies would optimize their performance. In agreement with previous studies (Palanca et al. 2013, Scheidler et al. 2015), we here obtained further evidence that *Drosophila* species can differentiate between different yeast species based on chemosignals. However, our data suggest that *Drosophila* flies do not necessarily prefer the yeast species associated with their natural habitat and that the attractiveness of a yeast species correlates instead with the number of attractive volatiles in the headspace of that yeast culture (Fig. 2B, Supplementary material Appendix 1 Fig. 3B–D). Here, a summation of the activity of attraction-mediating channels in the olfactory system might have led to an enhanced attractiveness of the respective yeast species. Nevertheless, additional experiments still need to be conducted with a broader selection of yeast species from inside and outside of *Drosophila* ecological niches to make further predictions about the general applicability of these observations.

The fact that different *Drosophila* species can be attracted to the headspace of a yeast that the flies most likely do not encounter in nature, indicates that the attraction to yeast-generated chemosignals is an evolutionarily shared trait across a wide array of *Drosophila* species (Scheidler et al. 2015, Becher et al. 2018, Günther et al. 2019).

Of our three tested yeast species, *V. humicola* was the one with the lowest level of attractiveness for each of our selected *Drosophila* species (Fig. 2B). In comparison to the other two yeasts tested, *V. humicola* produced noticeably fewer of the volatiles that are known to be generally attractive to *Drosophila* species (Supplementary material Appendix 1 Fig. A3B–D). As mentioned before, the production of attractive volatile compounds mediates yeast-insect interaction and promotes mutualistic relationships with *Drosophila* flies or other insects (Nout and Bartelt 1998, Becher et al. 2010, 2018, Christiaens et al. 2014). However, mushrooms are generally poor in nutrients and yeast species relying on this substrate as a host might not be able to invest resources into the production of chemosignals that are not byproducts of the yeast's necessary metabolic activity. Consequently, the habitat and associated yeast communities of *D. putrida* and other mycophagous *Drosophilidae* might be devoid of many attractive odorants. Therefore, yeast species emitting even only a few attractive volatiles could already stand out in the odor background, thus allowing that yeast to invest only minimal resources in the production of attractive chemosignals for successful insect dispersal. Ultimately, when comparing the

amount of attractive chemosignals in the headspace of our target yeast species, it is important to consider that all species were grown on a standard growth medium (yeast malt medium). Therefore, it is a possibility that, for example, *V. humicola* might emit a different odor profile when grown on a substrate closer to its natural conditions (Becher et al. 2018). In the future, additional studies should address the chemosignal profile of these yeasts in combination with different natural growth substrates, such as used in this study (e.g. banana, cactus and mushroom).

Drosophila preference for yeast species is based on a multi-chemosensory assessment

We found that the initial attraction of the three *Drosophila* species towards the headspace of the three yeast species differed from the final decision for yeast acceptance at oviposition sites of the female flies (Fig. 2). While the yeast preference in the trap assays fully depended on volatile cues, during oviposition behavior, *Drosophila* females had the opportunity to base their decision on both smell and taste. Interestingly, for *Drosophila* females we observed changes in the attractiveness of the different yeast species when the flies had the option to evaluate the properties of the yeasts using gustatory aspects (Fig. 2B, D). We conclude that *Drosophila* flies assess the attractiveness of a yeast species after evaluating multiple chemosensory information and that an initial attraction based solely on olfactory cues can be re-evaluated following close-range, gustatory input, as has been suggested previously (Karageorgi et al. 2017). In addition, for *Drosophila* flies, it has been shown that volatiles emitted by a yeast can mediate attraction and oviposition preference while host substrate volatiles play only a secondary role in these fly behaviors (Becher et al. 2012). We found that the combination of a familiar yeast and a naturally occurring host material has an impact on yeast preference and oviposition in *Drosophila* species that have a restricted host association (*D. mojavensis*, *D. putrida*; Fig. 2D, 3C–E, Supplementary material Appendix 1 Fig. A2). Especially for these *Drosophila* specialist species the preference of one yeast species over another is perhaps context dependent and is influenced by the substrate on which the yeasts grow (Fig. 2D, 3C–E). Thus, it appears that the simultaneous presence of yeast and host material synergistically stimulates the oviposition decision, possibly through the increased quantity of attractive odorants and other chemosignals perceived by the *Drosophila* females when host and yeast are combined. Additionally, the different yeast species might have processed their substrate in a species-specific manner, further enhancing the attractiveness of their growth medium as a species-specific oviposition site (e.g. detoxification of secondary metabolites, Fogleman et al 1986). Lastly, based on our data, we exclude the possibility that increased egg numbers in oviposition assays with host and yeast presence are a result of changes in the consistency of the presented substrate since we did not find a consistent preference of *D. mojavensis* or *D. putrida* for oviposition on the purée itself (Supplementary material Appendix 1 Fig. A1C–D).

Larval fitness benefits from association with yeast from natural habitats

Oviposition preference by our tested adult *Drosophila* species for various yeasts was not directly mirrored by the performance of their larvae (i.e. survivorship and developmental rate) with those yeasts. Although *Drosophila* females might prefer to oviposit in the presence of yeast species from different habitats, their larvae did not benefit from this new association, and this may represent an evolutionary constraint on adaptation to a novel niche. In fact, adult preference for yeast species that are not linked to the ecological niche of a *Drosophila* species over yeast from shared habitats might be fatal for the entire next generation, as seen by the example of *D. putrida* (Fig. 4). Contradictory to the assumption that females would seek oviposition sites that are optimally suited in nutrition and yeast community composition for high larval survivorship numbers and a fast larval development (preference–performance hypothesis, Jaenike 1978, Thompson 1988), previous studies (Mayhew 2001, Anagnostou et al. 2010a) and our present findings indicate that mother does not always know best. Mounting evidence suggests that insect females generally favor substrates as oviposition sites that they themselves prefer as a food source and which promote adult fitness (fecundity and fertility; Fig. 2D, 3C–E, 5), putting the needs of their offspring secondary to their own (Scheirs et al. 2000, Mayhew 2001, Anagnostou et al. 2010a). According to this hypothesis, higher numbers of eggs laid could outweigh possible losses in larval survivorship and disadvantages from the slower developmental rates of progeny. As an underlying principle for this 'bad-mother' behavior, a tradeoff between female fitness and fitness of their progeny has been proposed, making adult performance on host material the predominant factor influencing adaptation (Mayhew 2001, Anagnostou et al. 2010a). Furthermore, differences between larval and adult performance in association with a certain yeast species are likely caused by differences in nutritional needs, which may be an explanation for distinct yeast preferences between developmental stages (Cooper 1960). In addition to previous hypotheses such as the 'bad mother' hypothesis, we propose that differences in yeast preference between adults and larvae in turn could reduce cross-generational competition for nutrition and resources in *Drosophila*.

How did the presence of *S. cerevisiae*, a yeast species that serves as a food source for many *Drosophila* species, have such a negative impact on the survivorship rate of *D. putrida* larvae? We know little about the ecology of *D. putrida* other than that this *Drosophila* species is mycophagous, and we also know little of the nutritional demands of these fly progeny. Mushrooms generally only offer low amounts of sugars (Portabella mushroom per 100 g: approx. 2.5 g sugars (US Dept of Agriculture)), which makes them generally a bad resource for alcohol fermentation and thus alcohol levels found in mushroom are expected to be naturally rather low. The yeast *S. cerevisiae*, however, produces high amounts of alcohol while obtaining energy from the fermentation of

sugars found in its host substrates, such as fruits. We propose that the production of alcohol via *S. cerevisiae* in the diet led to levels toxic to *D. putrida* larvae that presumably only tolerate low concentrations of alcohol, as they would naturally not encounter high concentrations in their mushroom host and are expected to have low tolerance for ethanol. Experiments in the past have shown a general trend, with exceptions, correlating *Drosophila* ethanol tolerance to the suitability of their breeding sites for fermentation processes (David and Van Herrewege 1983). However, additional work is still needed to fully understand the nutritional and metabolic consequences of larvae reared on nutrient poor substrates, such as mushrooms.

The dietary value of yeast species differs for *Drosophila* species

Ovary size correlates with ovariole number and therefore determines potential female fecundity (Boulétreau-Merle et al. 1982, R'kha et al. 1997, Klepsatel et al. 2013, Mendes and Mirth 2016). Moreover, well-fed females with enlarged fat storage will withstand a shortfall of food resources longer than malnourished flies. Both weight and ovary size, are affected by nutritional conditions during the adult female's larval development (Hodin and Riddiford 2000, Tu and Tatar 2003, Green and Extavour 2014, Qiao et al. 2019). Our data indicate that the tested yeast species had differential dietary values for our three *Drosophila* species. We observed that a yeast species could enhance ovary and female size in one species, while there would be no beneficial impact on adult traits in other *Drosophila* (Fig. 5). Thus, each *Drosophila* species must gain different nutritional effects from the same yeast. At least in *D. melanogaster* and *D. mojavensis* the size of ovaries and weight of females of the next generation grown on our selected yeast species roughly correlated with the sequence of yeast preference at oviposition sites of the *Drosophila* females from the parental generation (Fig. 2D, 3C–E, 5B). This finding provides additional evidence that *Drosophila* females prefer yeast species and substrates that enhance their adult fecundity and fitness.

Drosophila flies and yeast together modify their ecological niche

In our study, we provide evidence that *Drosophila* flies and associated microorganisms accelerate the decomposition of their host material (Supplementary material Appendix 1 Fig. A5, A6). In agreement with previous studies our results suggest that *Drosophila* flies inoculate new host material with microorganisms such as yeast spores that are transferred both on the flies' bodies and/or through the oral-fecal route (Ganter 1988, Starmer et al. 1988, Stamps et al. 2012). According to our data and in agreement with observations from another study (Becher et al. 2012), we conclude that flies and microbes together broke down the host material faster than flies alone. We speculate that this accelerated decay is the result of activities associated with niche construction processes of fly and yeast. Both interaction partners (i.e. fly and yeast) likely released nutritional factors or enzymes that benefited their mutual performance through degradation of the host substrate. Furthermore, we found that the presence of flies and their microorganisms kept host samples from being overgrown by filamentous fungi, which further supports arguments towards joint fly–yeast niche construction efforts (Supplementary material Appendix 1 Fig. A6, A7). However, future research remains to be conducted to measure and confirm this proposal, including more detailed metrics associated with host substrate decay rates (e.g. CO_2 production, changes in sugar contents and volume or weight loss).

We expected that yeast species with a specialist lifestyle would outperform yeast species not commonly found in that habitat (e.g. cactophilic yeast performance would be optimized on cactus substrates). However, overall our data were not conclusive enough to evaluate yeast performance and growth on all tested host purées, and here, future work needs to be done to refine the assays used to assess these parameters in complex tritrophic interactions. Nonetheless, on banana purée it appears that *S. cerevisiae* converted available sugars faster than the yeast species that are naturally not found on this host (Supplementary material Appendix 1 Fig. A5). Additionally, yeast species naturally associated with a host were able to prevent or hinder the growth of molds on their respective host substrate (Supplementary material Appendix 1 Fig. A7), which is presumably linked to the competitive performance of that yeast species on its natural host. Additionally, yeast species naturally associated with a host were able to prevent or hinder the growth of molds on their respective host substrate (Supplementary material Appendix 1 Fig. A7), which is presumably linked to the competitive performance of that yeast species on its natural host. Our observational data is in agreement with Caballero Ortiz et al. 2018 who found that volatiles released by Drosophila associated yeasts affected the phenotype of exposed fungal molds and suppressed the initialization of the molds' chemical defense mechanisms. Moreover, the same study provides data that mortality rates of Drosophila larvae are drastically reduced once their associated yeasts disable the production of fungal insecticidal compounds (Caballero Ortiz et al. 2018).

Association with yeasts from new habitats as a chance for speciation in *Drosophila*

Our initial hypothesis predicted that the preference of *Drosophila* flies for yeast species from their native habitats would be well established and maintained to ensure mutual benefits for both interaction partners. However, based on our findings and recent work (Günther et al. 2019), we know now that certain *Drosophila*–yeast associations are less established than we initially assumed and that the flies are not only attracted to yeast from their own ecological niche but can even favor yeast species from completely different niches. Thus, at this juncture it remains unclear whether association of fly and yeast are coincidental, or evidence for the early stages of an evolving mutualistic interaction. The preference

of flies towards certain yeasts is mediated via chemosignals emitted by those yeasts that are universally attractive to *Drosophila* species, which are generally known for their shared preference for microbial fermentation. In agreement with Starmer (1981) and O'Conner et al. (2014), we suspect that *Drosophila* flies were capable of populating extremely diverse habitats as a result of an association with resident yeast communities that have unique physiological adaptations to their environment. We propose that the association of adult *Drosophila* flies with a new yeast species has the potential to allow the flies to adapt to new environments and that this insect-yeast association is a starting point for evolutionary processes leading to speciation events. For example, a *Drosophila* species might be able to have a more generalist host range if in turn its associated yeasts can perform well on a wide range of growing substrates (Fig. 6). However, one of the constraining factors for the adaptation of *Drosophila* to new habitats appears to be the larval performance in presence of a new yeast species and the performance of the larvae on a novel host substrate (Markow 2019). Our data suggest that *Drosophila* larvae are more sensitive to changes in the composition of yeast communities than adults, and a new association of fly, yeast and host can only evolve if juveniles can thrive in the new dietary situation.

Challenges arising from research on *Drosophila*–yeast interactions

Studies provide evidence that yeast preference can vary between and possibly even within *Drosophila* populations (Günther et al. 2019). Furthermore, not all isolates of a yeast species are equally attractive to flies from one *Drosophila* species (Palanca et al. 2013, Buser et al. 2014). Some yeast strains can lead to attraction behavior while other strains of the same species induce aversion or are neutral in their attraction of *Drosophila* flies (Palanca et al. 2013, Buser et al. 2014). It is also important to mention that all our tested *Drosophila* species have been kept as laboratory strains over an extended time-period, and it is possible that the observed preference and performance differs from the corresponding natural populations. Moreover, we acknowledge these limitations to our study and cannot exclude the possibility that individual results might differ when tested across several different *Drosophila* populations for our target species and across multiple strains of the three tested yeast species. However, we anticipate that our overall conclusion will remain the same, namely that the association of *Drosophila* species with beneficial yeasts from an overlapping habitat are less stable and less established than we initially predicted. We expect that experiments with natural, diverse populations of several *Drosophila* species will continue to support our hypothesis that there is seemingly no conserved aspect to specific *Drosophila*-yeast associations. Moreover, that certain chemosignals in the headspace of yeast species, perhaps in an additive manner, can determine the attractiveness of a given yeast species to the fly genus *Drosophila* as a whole. In future work, we would like to tackle exactly these questions with a wider range of *Drosophila* species (and populations) as well as with several strains of a broad range of yeast species, in order to determine whether effects are greater within or across these species. We consider this study as an attempt to understand the complexity of a tritrophic interaction, such as found between our model organisms, *Drosophila*, yeast and their shared host substrates, where we look at all interaction partners simultaneously. Furthermore, in our assays we tested novel approaches on documenting host decay, such as sugar content measurements (Supplementary material Appendix 1 Fig. A5), and worked with *Drosophila* as well as yeast species from drastically different habitats. We hope that our data sparks interest in the research community to work on all trophic levels at once, instead of individual, unidirectional approaches, where perhaps only the yeast or insect perspective is considered.

Speculations

The current publication and the enclosed data offers several novel directions to pursue. First, we speculate that the alimentary canal of *Drosophil*a flies acts as a filter for beneficial microbes and that the flies vector these associated microorganisms through their frass to new host sites. Here, we hypothesize that both, fly and yeast, together accelerate host decay in a shared effort, with mutual benefits. Furthermore, we provide data that yeasts suppress the growth of filamentous fungi, especially on their natural growing substrate (e.g. cactophilic yeast growing on cactus), which again, potentially provides benefits for both, fly and yeast. However, it remains to be determined whether this is the result of a mutualistic partnership, or a commensalism, with the *Drosophila* flies as beneficiaries, or just a coincidental convenience.

Second, we speculate that trans-generational competition for resources in *Drosophila* is reduced or avoided through differences in yeast preference between *Drosophil*a life stages. Thus, food sources for larvae and adults might be spatially separated (e.g. female feeding and oviposition preference may significantly differ). Lastly, we observe that larval stages of specialist *Drosophila* species are strongly affected by the presence of yeasts from different habitats. As a consequence, we propose that the host spectrum of a *Drosophila* species might correlate with their tolerance towards microbial pathogens (e.g. generalist species may have a higher immunity against a wide array of pathogens compared to specialist species).

Conclusion

In summary, our study provides evidence that after the vectoring of a yeast species by *Drosophila* flies to a new food source or breeding site, flies and yeast together potentially accelerate the breakdown of their host material while they simultaneously hinder the growth of filamentous, harmful fungi. Thus, we speculate that, flies and yeast both participate in activities of niche construction, while reproductive benefits for both insect and microbe seem to have initiated a partnership that

is mediated predominantly by chemosensory cues. However, future work should follow up on the question of whether the nature of *Drosophila*–yeast associations is contextual or a budding mutualism (Günther and Goddard 2019, Günther et al. 2019). Furthermore, the number of insect-attracting chemosignals released by a yeast species seemingly depends on the amount of nutrition found in the yeasts' natural growing substrate. We propose that yeasts associated with fruit or cacti, which are habitats rich in nutrition, can metabolically invest more resources into the production of attractive chemosignals for insect vectors, while yeasts from niches that are poor in nutrition, such as mushrooms or leaves (Starmer 1981), can only produce a few attractive cues. A broader assessment of insect attractants across differing yeast strains and species should be conducted to test this hypothesis.

In our experiments we found that yeast preference of *Drosophila* flies appears to be context dependent and is affected by the yeasts' growth substrate, an outcome also observed by Günther et al. (2019). Moreover, the level of larval performance of *Drosophila* flies in association with a yeast partner species depends on co-adaptation processes between fly and yeast, most likely due to exchange of nutrients. Our tested fly species had a higher reproductive success on substrates inoculated with a yeast species from their natural ecological niche, and on substrates where yeast and host substrate were provided together. Additionally, our results support the 'bad mother' hypothesis (Mayhew 2001), where *Drosophila* females do not necessarily appear to choose their oviposition sites in regards to an optimized larval performance. This observation further suggests that the attractiveness of a yeast species does not always correlate with a short-term fitness advantage for the attracted *Drosophila* species. Furthermore, we propose that in *Drosophila*, life-stage dependent changes in yeast preference could allow for a reduction in cross-generational resource competition between adults and larvae through spatial separation of food sources.

Opposite to our initial hypothesis, we found that adult *Drosophila* can be attracted to yeast species from profoundly different ecological niches, and that there is perhaps a broad acceptance for various yeast species across this insect genus. This may arise from the fact that the number of attractive odorants produced by yeast in our experiments correlates more strongly with yeast preference of *Drosophila* flies than a common habitat. Based on our results, we propose that the propensity for *Drosophila* adults to associate with yeast species from unfamiliar habitats can lead to adaptation of those flies towards new environments, which ultimately can drive speciation events, but only if the progeny of those flies can also survive this new host or yeast association. Consequently, we propose that successful novel associations with yeast from new habitats can promote *Drosophila* evolutionary events, such as adaptation to a new environment or host.

Data availability statement

All data supporting the findings of this study, including raw images, behavioral metrics, weight and size measurements, electrophysiological data and the results of chemical analysis are available from EDMOND (provided by Max Planck Society) <https://dx.doi.org/10.17617/3.3p>.

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Conflicts of interest – The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Author contributions – SK and IWK share first authorship. BSH and MK share senior authorship. SK and IWK generated the original hypotheses for this study, supported by BSH and MK Experiments were designed by SK in conjunction with IWK. Microbiological work was executed by SK, as were the measurements of ovary development, adult weight and survivorship tables. SK was responsible for all photographs and microscopy of raw data, with support from IWK. The attraction assays, oviposition and all other behavior data were collected and analyzed by SK, with support from IWK. Sugar content measurements were performed and analyzed by ME supervised by JG. All statistical analyses were performed by SK. Diagrams, illustrations and figures were created by SK and IWK, as was the original written manuscript with support from ME. Each author subsequently improved the final version of the manuscript as well as the figures, and all authors assisted with any revisions towards the final publication.

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Supplementary material (available online as Appendix oik-07180 at <www.oikosjournal.org/appendix/oik-07180>). Appendix 1.