

“This is not an Apple”–Yeast Mutualism in Codling Moth

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Abstract The larva of codling moth *Cydia pomonella* (Tortricidae, Lepidoptera) is known as the worm in the apple, mining the fruit for food. We here show that codling moth larvae are closely associated with yeasts of the genus *Metschnikowia*. Yeast is an essential part of the larval diet and further promotes larval survival by reducing the incidence of fungal infestations in the apple. Larval feeding, on the other hand, enables yeast proliferation on unripe fruit. Chemical, physiological and behavioral analyses demonstrate that codling moth senses and responds to yeast aroma. Female moths are attracted to fermenting yeast and lay more eggs on yeast-inoculated than on yeast-free apples. An olfactory response to yeast volatiles strongly suggests a contributing role of yeast in host finding, in addition to plant volatiles. Codling moth is a

widely studied insect of worldwide economic importance, and it is noteworthy that its association with yeasts has gone unnoticed. Tripartite relationships between moths, plants, and microorganisms may, accordingly, be more widespread than previously thought. It, therefore, is important to study the impact of microorganisms on host plant ecology and their contribution to the signals that mediate host plant finding and recognition. A better comprehension of host volatile signatures also will facilitate further development of semiochemicals for sustainable insect control.

Keywords Plant-insect-microbe-interaction · Mutualism · Herbivory · Chemical communication · Semiochemicals · Tortricidae · Lepidoptera

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Introduction

“*Ceci n’est pas une pomme*” (“*This is not an apple*”), a painting by René Magritte, shows an apple, which is an incomplete description of an apple, as indicated by its title. It is reminiscent of our past attempts to study host plant interactions in codling moth *Cydia pomonella* (Lepidoptera, Tortricidae) and to identify the chemical cues that mediate codling moth attraction to apple, by studying apple volatiles only (Witzgall et al., 2008). We now show that yeast, found in codling moth larval galleries, is an essential part of the larval diet and that egg-laying females of codling moth sense and respond to yeast volatiles.

Associations between yeasts and insect herbivores are widespread, and these interspecific interactions play a crucial role in yeast and insect evolution (Ganter, 2006; Janson et al., 2008; Klepzig et al., 2009; Herrera et al., 2010). However, mainly bark beetles, ambrosia beetles, drosophilid

flies, and social insects have been studied (Starmer and Fogleman, 1986; Farrell et al., 2001; Mueller et al., 2005; Ganter, 2006; Hofstetter et al., 2006; Davis et al., 2012), and there are but a few records of yeasts that have been found in the frass of lepidopteran larvae (Lachance et al., 1988; Rosa et al., 1992; Molnar and Prillinger, 2005), including codling moth (Listemann, 1988).

The search for yeasts in codling moth larval galleries has been fueled by current studies of *Drosophila melanogaster*, showing that the flies mainly use microbial, and not plant cues, to locate feeding and oviposition sites (Becher et al., 2012). In addition, flies are thought to employ two distinct types of chemosensory receptors to find adult and larval food sources: ionotropic receptors (IRs) and odorant receptors (ORs). Odorant receptors are tuned to plant volatiles and probably evolved during the transition to terrestrial life, while the ancestral IRs sense water-soluble compounds including microbial volatiles (Benton et al., 2009; Croset et al., 2010).

Ionotropic receptors also have been identified recently in the antennal transcriptome of codling moth *C. pomonella* (Bengtsson et al., 2012). A typical IR-encoded odorant is acetic acid (Silbering et al., 2011), which is a key compound of *Drosophila* attraction to yeast (Becher et al., 2010; 2012). In codling moth, acetic acid synergizes attraction to green plant volatiles, which are only weak attractants by themselves (Landolt et al., 2007; Knight et al., 2011). This led us to hypothesize the role of microorganisms in codling moth host finding.

Methods and Materials

Yeast DNA Isolation and Taxonomic Identification Apples (cvs. Aroma, Discovery and Delicious) infested with codling moth larvae were collected in orchards around Alnarp (Scania, Sweden) and Yakima (WA, USA) in July and August of 2011. Larvae ($N=25$) walked under sterile conditions for 5 min on YPD agar plates (20 g/L peptone, 20 g/L glucose, 10 g/L yeast extract, 20 g/L agar). In addition, larvae ($N=12$) were surface-sterilized by rinsing in 10 % NaOCl for 10 sec, followed by a rinse in 70 % ethanol solution and in autoclaved distilled water. Larvae then were cut open with a sterile razor blade under a sterile fume hood. A sterilized inoculating loop was used to gather the guts, and this was streaked onto a YPD agar plate. Three typical isolates were selected for taxonomic identification. Apple flowers (May) were dipped directly onto YPD plates ($N=10$) and undamaged apples (July) were wiped with an inoculating swab ($N=10$; Alnarp). Plates were incubated at 25 °C for 3 day and emerging colonies of microorganisms were re-isolated up to six times on fresh YPD plates to obtain pure colonies.

Yeast isolates were grown overnight in liquid YPD medium in an incubating shaker at 25 °C. The cells were spun

down (10,060 g, 2 min) and washed with water. 200 μ l of the lysis buffer (2 % Triton X-100, 1 % SDS, 0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8), 200 μ l of a 25:24:1-blend of phenol, chloroform, and isoamyl alcohol, and 100 μ l acid-washed glass beads were added to the pellet. The mix was vortexed for 10 min and 200 μ l of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) were added. The suspension was centrifuged for 10 min at 10,060 g and 10 μ l RNase A (10 mg/ml) were added to the aqueous phase and incubated for 45 min at 37 °C. The DNA was precipitated with 1 ml 96 % ice-cold ethanol and 1/10 volume of 3 M sodium acetate. The mixture was centrifuged for 10 min at 10,060 g at 4 °C. The pellet was washed with ice-cold 70 % ethanol, air-dried and resuspended in 40 μ l TE buffer (pH 8). Concentration of genomic DNA was measured on NanoDrop.

The internally transcribed spacer (ITS) region of rDNA and the D1/D2 domains of large ribosomal subunit (LSU) were amplified by using ITS1/ITS4 and NL1/NL4 primer pairs, respectively (White et al., 1990; Kurtzman and Robnett, 2003). The polymerase chain reaction was run under the following conditions: 94 °C, 5 min followed by 30 cycles of 95 °C for 30 s, 50 °C for 45 s, and 72 °C for 45 s with the extension step of 72 °C for 10 min. The PCR products were purified using GeneJET PCR Purification kit (Fermentas, cat. no K0702) and sequenced using PCR primers by MWG Operon (Ebersberg, Germany). The sequences were identified by comparison to GenBank database of non-redundant sequences using BLAST (Altschul et al., 1990). Sequences were determined with an ABI 3730 gene analyzer.

Larval Feeding Assays Unripe apples (cv. Delicious) from an unsprayed orchard (Moxee, WA, USA) were sterilized in 5 % NaOH for 30 min, and then consecutively rinsed and dried, with 70 % EtOH and autoclaved distilled water. *Metschnikowia andauensis* was grown on YPD medium. Half of the sterilized apples were dipped five times into a 500-ml solution of yeast and placed on a paper towel in a fume hood to dry. A gelatin capsule was attached with paraffin on the shoulder of each apple. One end of the capsule was cut-off with a razor blade, and the second part of the capsule was then slid over the cut end. One sterilized black-headed codling moth egg on a <15 mm² piece of wax paper was placed inside each gelatin capsule and touching the fruit. Apples were placed inside 350-ml clear plastic cups closed with a lid. Cups were placed in a room maintained at 22 to 25 °C for 35 day. Assays were conducted on five dates with 10 apples per treatment.

After 35 day, each apple was cut in half to expose the feeding galleries, and larvae were scored as alive or dead. A sterilized inoculating metal loop was used to swab the larval gallery and to inoculate a YPD agar plate. Two swipes were made from each gallery. Sub-samples also were collected from the skin of the apple enclosed inside the gelatin capsule, the

apple skin outside the capsule, the calyx of the fruit, and from the seed area of uninfested fruit. Plates were incubated for 2 day, followed by a visual determination of the presence of yeast colonies based on macromorphology.

Oviposition and Upwind Attraction Behavioral Assays Codling moths were reared on an artificial wheat germ-based diet. Adults were kept in 33×33×33-cm Plexiglass cages under a 16:8 L:D photoperiod (65±5 % RH, 22 °C). Females were mated 1 day after eclosion and used for wind tunnel and oviposition experiments on the following day. Adults used for behavioral tests were negative for the presence of *Metschnikowia* yeasts, according to females that were allowed to walk for 5 min on YPD agar plates ($N=25$).

Single mated females were placed in Plexiglass cages containing 2 apples in the center, spaced at 15 cm. Green apples (cv. Discovery) were sterilized (see above). A hole (ca. 2 mm×3 cm) was punched into the apples (control), and one *M. andauensis* colony from an agar plate was thoroughly spread in this cavity, using a Pasteur pipette (yeast treatment). In a second test, apples infested with codling moth larvae (3rd to 5th instar) were placed side by side with undamaged apples. The number of eggs laid on the apples was counted after 48 h. Presence of yeasts in the larval galleries was verified after the test.

Females were flown in a wind tunnel (180×90×60 cm), illuminated from above at 2–3 lx (Witzgall et al., 2001). Incoming air (30 cm/s, 22 to 24 °C, 50 to 60 % RH) was filtered with active charcoal. Insects were placed in 2.5×12.5-cm glass tubes closed with gauze and kept in the wind tunnel room for 1 h before testing. They were tested individually between 1 and 4 h after onset of the scotophase, in five batches of 10, and the following types of behavior were recorded: flight initiation (take-off from holding tube) and upwind flights over 120 cm towards the odor source. The odor source was liquid minimal growth medium (Merico et al., 2007) inoculated with *M. andauensis* 18 h before onset of the tests. A gentle charcoal-filtered airstream (0.1 L/min) passed through the medium in a wash bottle and was led through a teflon tube (6 mm ID) into the wind tunnel. Control tests were done with growth medium without yeast.

Collection of Fermentation Volatiles and Chemical Analysis Fermentation of *M. andauensis* and *M. pulcherrima*, respectively, in 1 L minimal growth medium (Merico et al., 2007) was done in bioreactors (Multifors/Infors HT 1.4 L, Bottmingen, Switzerland) set to 25 °C, pH 5 and an airflow of 1 L/min, maintaining the dissolved oxygen concentration at >30 % of saturation. Headspace was collected between 24 and 48 h after inoculation with air filters (Super Q, 80/100 mesh; Alltech, Deerfield, IL, USA; Bengtsson et al., 2001). The filters were eluted with 300 µl of hexane, and the filter extracts were stored in glass capillary tubes.

Samples were analyzed on a combined gas chromatograph and mass spectrometer (GC-MS; 6890 GC and 5975 MS, Agilent Technologies, Palo Alto, CA, USA), operated in splitless injection and electron impact (EI) ionization mode at 70 eV. The GC was equipped with fused silica capillary columns (30 m×0.25 mm, $df=0.25$ µm), DB-Wax (J&W Scientific, Folsom, CA, USA) or HP-5MS (Agilent Technologies). Helium was used as the mobile phase at an average linear flow rate of 35 cm/s. Two µl of each sample were injected (splitless mode, 30 sec, injector temperature 225 °C). The GC oven temperature was programmed from 30 °C (3 min hold) at 8 °C/min to 225 °C (5 min hold). Compounds were identified according to their retention times (Kovat's index) and mass spectra, using a NIST library (Agilent) and with authentic standards.

Electrophysiology Antennal responses of *C. pomonella* females to yeast volatiles were studied by combined GC and electroantennographic detection (GC-EAD), using an Agilent 6890 GC and an electroantennogram (EAG) apparatus (IDAC-2; Syntech, Kirchzarten, Germany) for data acquisition. The GC columns and the temperature programs were the same as for the GC-MS analysis. Hydrogen was used as the mobile phase at an average linear flow of 45 cm/s. At the GC effluent, 4 psi of nitrogen were added and split 1:1 in a Gerstel 3D/2 low dead volume four-way-cross (Gerstel, Mülheim, Germany) between the flame ionization detector (FID) and the EAD. The GC effluent capillary for the EAD passed through a Gerstel ODP-3 transfer line, that tracked GC oven temperature, into a glass tube (30 cm×8 mm), where it was mixed with charcoal-filtered humidified air (18–20 °C, 50 cm/s). The antenna was placed 0.5 cm from the outlet of this tube.

Two- to 3-d-old mated females were used for antennal recordings. The antenna was cut at the base and inserted into the recording glass electrode filled with Beadle-Ephrussi Ringer and connected to a pre-amplifier probe connected to a high impedance DC amplifier interface box (IDAC-2; Syntech). The reference electrode was connected to the antennal tip after cutting the distal segment.

Results

Occurrence of Yeasts in Codling Moth Larval Galleries Figure 1 shows a fresh and a deserted codling moth *C. pomonella* larval gallery. All larvae extracted from field-collected apples in Sweden carried two yeasts, which were identified from ITS and D1/D2 rRNA gene sequence analysis as *Metschnikowia andauensis* and *M. pulcherrima* (Ascomycota, Saccharomycetes). *Metschnikowia pulcherrima* was also found in the guts of codling moth larvae that had been collected in Washington orchards, USA. In Sweden, *M. andauensis* and *M. pulcherrima* were found in

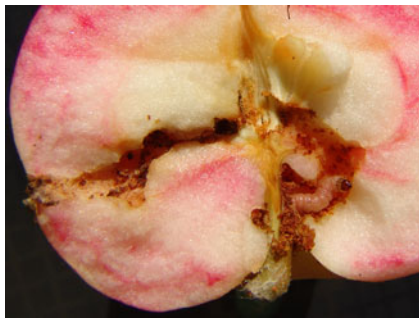


Fig. 1 Two codling moth *Cydia pomonella* galleries, deserted (*left*) and with larva (*right*). The larva does not range the entire apple, but builds voluminous galleries that contain *Metschnikowia* yeasts. Larval galleries, fresh and abandoned, become rarely infested with fungi

all apple flowers samples, and *M. andauensis* also was present in few skin samples of undamaged apples.

Species identification is tentative because there are relatively few nucleotide differences between species of the *M. pulcherrima* clade (Lachance, 2011), and some nucleotides in the divergent regions of ITS and D1/D2 were poorly resolved, suggesting that the isolates may be hybrids.

Occurrence of other microorganisms was not consistent, these were not found on all larvae and there was no overlap between Sweden and USA. These species included the yeast *Cryptococcus tephrensis* (Basidiomycota, Tremellomycetes) and the yeast-like fungus *Aureobasidium pullulans* (Ascomycota, Sordariomycetes) in Washington and the bacterium *Pantoea agglomerans* (Proteobacteria, Enterobacteriales) in Sweden.

In the laboratory, galleries of codling moth larvae feeding on sterilized apples did not contain yeasts. In stark contrast, all feeding galleries in apples that were dipped in yeast prior to larval infestation were positive for yeast ($N=50$). The skin of apples dipped in yeast broth was positive for yeast after 35 day, but the interior cavity of yeast-treated fruits without codling moth larvae was negative for yeasts.

Effect of Yeast on Codling Moth Larval Development Presence of *M. andauensis* yeast in larval galleries strongly synergized codling moth development (Fig. 2a). Larval mortality was significantly lower on sterilized apples that had been dipped into a yeast broth than on sterilized apples without yeast; larval development was accelerated in the presence of yeast and more larvae pupated; and yeast decreased the incidence of mold during a test period of 35 day ($P<0.05$, $P<0.005$ and $P<0.001$, respectively; Fisher's exact test; Fig. 2a). Without yeast treatment, mold was growing inside all apples that contained dead larvae. In the field, even deserted feeding galleries rarely become infested with molds (see also Fig. 1).

Attraction and Egg-Laying Response to Yeast Volatiles Since *M. andauensis* enhanced larval survival on apples, we

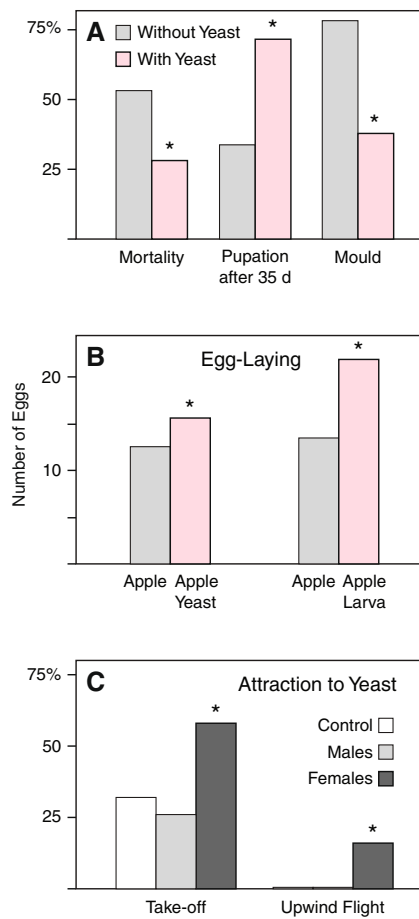


Fig. 2 a Codling moth *Cydia pomonella* larval mortality, larval development (number of larvae and pupae) and incidence of mould in larval galleries, during 35 day on sterilized apples, that were inoculated with yeast or left untreated ($N=50$). **b** Number of eggs laid on sterilized apples, with and without yeast inoculation (*left*) and on undamaged apples and apples infested with codling moth larvae ($N=50$). **c** Wind tunnel response of codling moth *C. pomonella* males and females ($N=90$) to *Metschnikowia andauensis* volatiles and to minimal growth medium. Asterisks show significant differences at $P<0.05$

investigated the behavior of mated codling moth females in response to this yeast. In a dual choice test (Fig. 2b), females laid more eggs on sterilized apples when a mechanically produced cavity was inoculated with *M. andauensis* ($P<0.05$, $t=2.562$, $df=58$; paired *t*-test). The difference was more pronounced when egg-laying females had the choice between control (mechanical injury alone) and an apple infested with a codling moth larva ($P<0.001$, $t=4.215$, $df=55$; paired *t*-test; Fig. 2b). Yeast inoculated into mechanically injured apple did not proliferate in the absence of larvae since the injury dried out after a few days.

The increased oviposition response was obviously due to additional yeast volatiles emanating from the apple. In order to corroborate the behavioral effect of yeast volatiles, we investigated the behavioral effect of *M. andauensis* headspace in a flight tunnel (Fig. 2c). Significantly more females

than males became activated and left the holding cage by flight in response to yeast volatiles ($P < 0.05$, $t = 3.051$, $df = 8$; t -test) and only females flew towards the source. However, the females did not closely approach or land on the odor outlet. Minimal medium alone did not elicit an upwind flight response ($P < 0.05$; Fisher's exact test; Fig. 2c).

Yeast Volatile Analysis The fermentation headspace of *M. andauensis* and *M. pulcherrima* on minimal medium contained 19 compounds that produced an antennal response in codling moth females. The yeasts produce volatile blends of similar composition, in different proportions (Table 1). The compounds, including phenols and terpenoids, were produced *de novo* from precursors in the minimal growth medium. Most

Table 1 Headspace volatiles from *Metschnikowia andauensis* and *M. pulcherrima* yeasts on minimal growth medium, 24 to 48 h after inoculation (mean relative proportion; $N = 3$) that elicited an antennal response in codling moth *C. pomonella* female antennae ($N = 6$)

Compound ^a	CAS ^b	KI ^c	<i>M. andauensis</i> 24–48 h	<i>M. pulcherrima</i> 24–48 h
Isobutyl acetate*	110-19-0	1004	tr ^d	– ^e
Butylacetate	123-86-4	1062	0.1±0.1	0.2±0.1
Isoamyl acetate*	123-92-2	1112	20.6±22.7	4.5±2.9
n-amyl acetate*	628-63-7	1165	0.3±0.2	0.9±0.4
2-heptanone*	110-43-0	1172	1.4±1.6	4.9±0.9
3-methyl-3-butenol*	763-32-6	1238	4.8±3.7	16.1±11.8
2-octanone	111-13-7	1275	–	0.7±0.2
Butyl-isothiocyanate	592-82-5	1306	1.5±2.3	0.1±0.1
Sulcatone*	110-93-0	1326	0.6±0.5	0.4±0.5
2-nonanone*	821-55-6	1380	0.3±0.4	3±0.3
Nonanal*	124-19-6	1385	7.0±5.2	0.8±0.2
Unknown		1620	0.1±0.1	22±0.9
Phenyl acetaldehyde*	122-78-13	1629	0.1±0.1	0.1±0.1
Unknown		1680	16.3±16.9	44±2.6
Geranial	141-27-5	1722	tr	–
Citronellol*	106-22-9	1755	0.9±0.4	1±0.1
2-phenylethyl acetate*	103-45-7	1804	0.5±0.7	–
Geranylacetone*	3796-70-1	1845	0.5±0.2	–
2-phenylethanol*	60-12-8	1896	43.1±26.7	1.4±1.6

^a Compounds marked with an asterisk were identified according to retention times on two columns (DB-Wax, HP-5) and mass spectra in comparison with synthetic standards. The other compounds were identified according to mass spectra using a NIST library and published Kovats retention indices (KI). All compounds listed were absent in control fermentations ($N = 3$), using minimal growth medium alone

^b Chemical abstracts service (CAS) registry number

^c Kovats retention index

^d Trace amounts

^e Not detected

of them have been identified from yeasts and other microorganisms (Swiegers et al., 2005; Schulz and Dickschat, 2007; Hernandez-Orte et al., 2008).

Discussion

Codling Moth Larvae are Associated with *Metschnikowia* Yeasts All larvae of codling moth *C. pomonella* collected in apple orchards carried *Metschnikowia* yeasts, and the results of our study suggest a mutualistic interaction between codling moth and these yeasts. Two species, *M. andauensis* and *M. pulcherrima*, were found in Sweden, and one of them, *M. pulcherrima*, also was found in Washington, USA. Yeasts were present in larval galleries and in the alimentary tract of codling moth larvae.

Larval feeding sustained yeast growth on apple, since yeast inoculated into mechanically injured apple did not proliferate in the absence of larvae. Feeding assays in the laboratory demonstrated that *M. andauensis* had a beneficial effect on codling moth larvae, by accelerating development and by reducing mortality. Yeast not only contributed to the larval diet, it also reduced the incidence of detrimental fungal infestations (Fig. 2a), *Metschnikowia* yeasts are efficient biocontrol agents of fungal fruit diseases (Kurtzman and Droby, 2001; Spadaro et al., 2008; Manso and Nunes, 2011).

An open question is whether codling moth larvae feeding on fruits other than apple also are accompanied by *Metschnikowia* yeasts. Other microorganisms were also found on some codling moth larvae in apple, but their occurrence was less consistent. This compares to bark beetles, which live in association with a consortium of microorganisms, including yeasts and other fungi (Davis et al., 2011).

The association of *M. pulcherrima* and codling moth is not new to literature (Listemann, 1988), and *M. pulcherrima* commonly occurs in apple (Bowen and Beech, 1964; Pelliccia et al., 2011). *Metschnikowia pulcherrima* also forms a symbiosis with the lacewing *Chrysoperla rufilabris*, where it is consistently found in the gut of adult insects (Woolfolk and Inglis, 2004). *Metschnikowia andauensis* and related species also have been found in larval excrements of European corn borer and corn earworm moths (Molnar and Prillinger, 2005). Other *Metschnikowia* yeasts are found with beetles from several families, lacewings, and drosophilid flies (Ganter, 2006; Nguyen et al., 2006; Yaman and Radek, 2008).

Several *Metschnikowia* yeasts associated with flowers are vectored by non-pollinating insects, especially nitidulid beetles and drosophilid flies. Both the formation of needle-shaped ascospores (Lachance et al., 2001, 2005) and the production of volatiles that attract insects (Landolt et al.,

2006; El-Sayed, 2012), which are also perceived by codling moth antennae (Table 1), are indicative of a close evolutionary relationships between *Metschnikowia* yeasts and insects.

Drosophila-yeast interactions are viewed as mutualistic (Starmer and Fogleman, 1986): yeasts provide an essential part of the larval diet (Nasir and Noda, 2003; Becher et al., 2012) and the flies promote long-distance dispersal of the yeast to suitable substrates, which increases the opportunity for outbreeding (Reuter et al., 2007). Mutualistic interspecific interaction eventually gives rise to interspecific communication: the sophistication of yeast-to-insect chemical communication is highlighted by a yeast infesting honey bee pollen, which mimics honey bee alarm pheromone, to attract a hive beetle. The beetle facilitates yeast proliferation inside, and dispersal outside bee colonies (Torto et al., 2007).

Yeast Volatiles Mediate Codling Moth Behavior Yeast fermentation headspace elicited upwind orientation flight behavior in codling moth females, which laid more eggs on apples inoculated with yeast (Fig. 1b, c). This points towards a contributing role of yeast volatiles in finding larval host plants. Both grapevine moth *Lobesia botrana*, a typical herbivore, and the fruit fly *D. melanogaster* show a strong attraction response and oviposition preference for yeast-infested grapes (Tasin et al., 2011; Becher et al., 2012). The flight response of codling females to yeast in the wind tunnel (Fig. 1c) compares to the flight response obtained with apples that provide odor and also visual cues (Reed and Landolt, 2002). In contrast, by using an invisible odor source, we did not manage to observe upwind flights in codling moth females towards apple volatiles in earlier experiments (e.g., Ansebo et al., 2004; Coracini et al., 2004).

Fermentation volatiles may, in addition to larval host plants, also signal adult food sources. Authentic and synthetic yeast odor blends attract a great many moths including codling moth (Dethier, 1947; El-Sayed et al., 2005; Landolt et al., 2011); these moths use many different host plants, and are accordingly not attracted by the same chemical cues for egg-laying. Even floral insect attractants, including 2-phenylethanol or phenyl acetaldehyde (Landolt et al., 2006; El-Sayed et al., 2008) are typical yeast odors (Table 1; Hernandez-Orte et al., 2008).

Another consequence of insect attraction to yeast is yeast transmission. In *C. rufilabris* lacewings, which are associated with *M. pulcherrima*, adults acquire the yeast from the environment, as there is no evidence of vertical transmission (Woolfolk and Inglis, 2004). This explains lacewing attraction to yeast volatiles (Table 1; Toth et al., 2009). *Metschnikowia* yeasts frequently occur in nectar (Pozo et al., 2011), and we found *M. andauensis* and *M. pulcherrima* in apple flowers. The onset of the first codling moth flight is tightly correlated with apple flowering (Bradley et al., 1979), which would provide a source of yeast inoculum.

Plant-derived volatiles known to attract codling moth females, such as pear ester, β -farnesene, or (*E*)-4,8-dimethyl-1,3,7-nonatriene are synergized by acetic acid (Landolt et al., 2007; Knight et al., 2011), which is a typical yeast metabolite. Acetic acid was not produced by *Metschnikowia* growing on minimal medium (Table 1), but a range of other *Metschnikowia* volatiles were perceived by codling moth female antenna, including compounds that are behaviorally active in other insects, for example nonanal, phenyl acetaldehyde, or 2-phenylethanol (Table 1; El-Sayed, 2012). It is still unclear which plant and yeast signals mediate codling moth host finding and oviposition, and which of the bioactive compounds are perceived via the OR and IR olfactory subsystems (Silbering et al., 2011; Bengtsson et al., 2012).

Attractant signals from yeasts are a resource for the behavioral manipulation of egg-laying female moths, since this communication channel is chemically distinct from the pervading background of green plant volatiles. This background odor interferes with field attraction of moths to plant volatiles (Knudsen et al., 2008), which is an obstacle for their widespread practical use (Coracini et al., 2004; Loeb et al., 2011; Knight and Light, 2012). Codling moth is controlled by pheromone-mediated mating disruption on 200,000 ha worldwide, but insecticide sprays are still a necessity at high population densities. Development of powerful attractants for reliable monitoring or for mass-trapping of codling moth females would greatly promote areawide use of pheromone-based control (Witzgall et al., 2010).

Microbial Mutualism and Evolutionary Diversification of Insect Herbivores The diversification of several phytophagous insect lineages has been linked with associated microorganisms and their effect on host plant ecology (Janson et al., 2008). *Metschnikowia* yeast clearly enhanced codling moth larval development, by providing nutritional services and by reducing adverse fungal infestations (Fig. 2a). Yeast may accordingly facilitate feeding on apple of different phenological stages, from unripe to fully grown mature apples, and may possibly contribute to colonization of other, taxonomically unrelated hosts, such as walnut.

Another important effect of microorganisms on insect-plant interactions is that they contribute with behaviorally active compounds to the bouquet of plants. Host shifts, which essentially contribute to the evolutionary diversification of insect herbivores (Fordyce, 2010), are probably to a large extent mediated by the chemical similarity between old and new hosts (Dres and Mallet, 2002; Smadja and Butlin, 2009). A showcase example of such a chemosensory-mediated host plant shift is the apple maggot *Rhagoletis pomonella* (Diptera: Tephritidae) sibling species complex, where the compounds that encode recognition and host finding have been identified by chemical analysis and behavioral studies (Linn et al., 2003; 2005a, b). Interestingly, several of the compounds that mediate

attraction of *Rhagoletis* fruit flies to fruit of their respective hosts apple, hawthorn, and flowering dogwood are known to be produced by fungi and yeasts, such as isoamyl acetate, ethyl acetate, 3-methylbutan-1-ol, and 1-octen-3-ol (e.g., Swiegers et al., 2005; Schulz and Dickschat, 2007; Hernandez-Orte et al., 2008). Yeasts or other microorganisms may facilitate host shifts by increasing the chemical and nutritional similarity between plants.

Our discovery that yeast metabolites contribute to host finding behavior in a typically herbivorous moth has consequences for ongoing research on how moths recognize and find their hosts. The interaction between yeast and plant volatiles in host finding behavior, including the coding of IR and OR ligands by the insect olfactory system and the identification of behaviorally active compounds, is a current research challenge. The knowledge generated by this work will facilitate the further development of environmentally safe semiochemicals for population monitoring and control of codling moth and other insects.

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