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

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Chapter 5

Spatial coupling of food and mates in Drosophila

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Abstract

In many taxa, individuals aggregate at food resources. This generates a co-localization of foraging and mating opportunities, which potentially promotes assortative mating. Here, we show that food and mates are spatially coupled in the fruit fly, *Drosophila melanogaster,* and we also explore the sensory and behavioural mechanisms underlying this co-localization. We track the mating location of flies in environments containing heterogeneous food patches and observe that *D. melanogaster* and several of its sibling species generally choose to mate on patches containing yeast - the primary diet of fruit flies. *D. melanogaster* is an exception with virgins primarily mating away from yeast, but previously mated females re-mating on yeast. Flies either alone or in pairs with another sex locate on yeast at night, but individuals' tendencies to be on the yeast during the day depend on several variables including sex, light conditions and presence of another sex. Mating location preference involves attraction to yeast-derived chemical cues (the combination of acetic acid and protein) and is modulated by the male-derived sex peptide received by females during mating. Preference for mating on yeast-containing patches is stronger at night than during the day, and increases with the passing of time since the first mating. We also find that *D. melanogaster* pairs exert preferences for mating on certain yeast species over others when exposed to patches containing different yeasts. Our study demonstrates a mechanism by which divergence in food preference can directly lead to assortative mating.

Introduction

Finding food and mates is crucial for survival and reproduction. Food resources and mates are often at different locations, making it a challenge for individuals to locate both of them in a complex environment. This challenge is alleviated when individuals aggregate at food resources, as observed in a variety of taxa including many herbivorous and frugivorous insects like beetles, moths, aphids, bees and flies (reviewed in Thornhill and Alcock, 1983) and fishes such as threespine stickleback and ricefish (Snowberg and Bolnick, 2012; Kakioka et al., 2021). In many insect species, including Hemiptera, Coleoptera, Lepidoptera and Diptera, males encounter and mate with females on host plants, where females emerge, forage or oviposit (Thornhill and Alcock, 1983; Berlocher and Feder, 2002). Host plants often stimulate the production and release of pheromones in insects, which attract the other sex or both sexes (Lin et al., 2015; Mercier et al., 2018; reviewed in Landolt and Phillips, 1997). Insect pheromones and host plant volatiles thus signal the presence of potential mates and food sources, leading to the aggregation of individuals (reviewed in Xu and Turlings, 2018).

When mates are spatially coupled with food, assortative mating can arise as a by-product of food choice since individuals that forage on similar food resources are more likely to encounter each other. In this scenario, food choice can become a magic trait which mediates both divergent adaptation and assortative mating (Servedio et al., 2011). This route is viewed as one of the most straightforward mechanisms of sympatric speciation (Rice, 1987). Evidence for this mechanism comes from a variety of insect taxa, such as the apple maggot fly (*Rhagoletis pomonela*) (Feder et al., 1994; Feder, 1998), pea aphids (*Acyrthosiphon pisum*) (Caillaud and Via, 2000) and cactophilic flies (*Drosophila mojavensis*) (Heed, 1978; Fogleman and Danielson, 2001). There are also examples where assortative mating is partially maintained by food choice. For instance, in threespine stickleback and stem-galling tephritid fly, both spatial segregation by food choices and mating preference contribute to the food choice-mediated assortative mating (Craig et al., 1993; Snowberg and Bolnick, 2012). Though mates are spatially coupled with food resources in a range of taxa, the proximate mechanisms that generate the spatial coupling of food and mates are poorly understood, limiting our understanding of the conditions that facilitate resource-mediated assortative mating.

The locations of foraging and mating may change based on individuals' physiological states such as mating status, sex, as well as environmental factors such as light intensity and times of the day or season (Thornhill and Alcock, 1983; Warburg and Yuval, 1997; Kaspi and Yuval, 1999; Raghu and Clarke, 2003; Stamps et al., 2005). Many species prefer to feed on different resources after mating. For instance, female fruit flies (*Drosophila melanogaster*) develop strong preferences for yeast as a food source only after mating (Ribeiro and Dickson, 2010). Compared with virgins, mated female cotton leaf worms (*Spodoptera littoralis*) prefer the green leaves of cotton (*Gossypium hirsutum*) over the flowers of lilac (*Syringa vulgaris*) (Saveer et al., 2012). Likewise, mated females in the two-spotted crickets (*Gryllus bimaculatus*) prefer protein-rich food compared with virgin females (Tsukamoto et al., 2014).

Sex differences in feeding sites exist in a variety of species, including several of the examples mentioned above. In *D. melanogaster*, mated females rather than males are strongly attracted to yeast for feeding after 3 days of yeast deprivation (Ribeiro and Dickson, 2010). Mated female *S. littoralis* prefer the foliage of cotton *Gossypium hirsutum*, while mated males prefer the flowers of lilac *Syringa vulgaris* (Saveer et al., 2012; Kromann et al., 2015). Field observations of Mediterranean fruit flies (*Ceratitis capitata*) reveal that females have more diverse diets than males since females forage on fruits, leaf surfaces, and fresh bird faeces, while males feed on fruits (Hendrichs et al., 1991; Warburg and Yuval, 1997). Light conditions are also a major factor for foraging as several species spend more time on food resources at night than during the day (e.g., tobacco fly, Mediterranean fruit fly and fruit fly) (Warburg and Yuval, 1997; Raghu and Clarke, 2003; Stamps et al., 2005). Moreover, light intensity modulates male location in Mediterranean fruit flies. When light intensity is high, in the late morning and early afternoon, males aggregate and perch on the bottom surface of leaves to attract females for mating, as the light intensity under the chosen leaves is much lower than under sunlit leaves (Kaspi and Yuval, 1999). This suggests that an individual's location changes based on light conditions. These examples show that the spatial locations of foraging and mating are affected by sex, mating status and light conditions. Therefore, to understand the long-term eco-evolutionary consequences of the spatial coupling of food and mates, the mechanisms underlying these dynamics must be understood.

In this study, we employ *D. melanogaster* to explore the spatial coupling of food and mates and its underlying sensory and behavioural mechanisms. *D. melanogaster* has become a powerful model organism for studying not only genetics and development but also behaviour and speciation (reviewed in Anholt et al., 2020; Wang et al., 2021). It is considered to be a dietary generalist, feeding on a broad range of food resources and occurring in most parts of the globe (Markow and O'Grady, 2008). However, some *D. melanogaster* populations do exhibit resource-associated differentiation (Capy et al., 2000; Wang et al., 2022). For instance, an urban population of *D. melanogaster* collected from a brewery in Brazzaville was found to be about twice more tolerant to ethanol – yeast fermentation products than a countryside population, and displayed a preference for mating with urban individuals (Capy et al., 2000). This imperfect generalist state of *D. melanogaster*, together with the wealth of tools that are available for mechanistic studies, make it a great model organism to study the sensory and behavioural mechanisms that mediate the spatial coupling of food and mates.

D. melanogaster is typically found on rotting fruits, but it is actually the yeast, which metabolizes the fruit sugars, that attracts adults and serves as the main food source for the flies (Becher et al., 2012). It provides the essential proteins, micronutrients and sterols which are absent in fruits but required for the growth and fecundity of flies (Baumberger, 1919; Cooke and Sang, 1970; Carvalho et al., 2010; Becher et al., 2012; Piper et al., 2014). Without yeast, fly larvae cannot develop into adults (Baumberger, 1919). Yeast stimulates the fly's fecundity by facilitating the egg-chambers to develop into eggs (Terashima and Bownes, 2004). Yeast also elevates the mating frequency of females (Gorter et al., 2016). *D. melanogaster* is found on different yeast species distributed in different habitats and locations

(Kurtzman et al., 2015). The heterogeneity generated by the differences in spatial distribution between yeast species provides a pronounced opportunity to explore resource-mediated differentiation between fly populations.

Here, we provide flies with different substrates and explore whether mating is spatially coupled with yeast, taking into account the flies' mating status, light conditions and the time since flies enter the mating arenas. We also examine whether flies prefer to mate on one yeast species rather than another. We find that *Drosophila* prefers (re)mating on yeast substrate, and that this preference is not an immediate by-product of spending most time on such substrate. Using mutant flies lacking sex peptide (males; *SP*) and sex peptide receptor (females; *SPR*), we find that *SP* and *SPR* mediate mating location differences between virgin and mated females. The combination of acetic acid and peptone attracts flies to remate on yeast and choice experiments reveal that *D. melanogaster* prefers mating on *Candida boidinii* over *Candida californica*. Together, our study experimentally demonstrates the preference of (re)mating on yeast in *Drosophila* and the mechanisms by which divergence in food preference can directly lead to mating preference.

Materials and methods

Drosophila stocks and fly rearing

Seven wild-type *D. melanogaster* strains were used in this study: three (*Canton-S* (*CS*), *Oregon-R* (*OR*), w^{1118}) were lab strains and the other four (*Ithaca* (*I16*), *Netherlands* (*N01*), *Tasmania* (*T01*), *Zimbabwe* (*Z*), kindly provided by Andrew Clark (Cornell University)) were originally sampled from Ithaca, NY USA; Netherlands, Europe; Tasmania, Australia; Zimbabwe, Africa, respectively (Grenier et al., 2015). Wild-type lab strains of other *Drosophila* species included *Drosophila simulans*, *Drosophila suzukii*, *Drosophila yakuba*. To investigate the possible role of sex peptide in mating location preference, we generated sex peptide null and control males by crossing *+;+;*SP^0*/TM3,Sb;+* females with *+;+; Delta130/Tm3,Sb;+* males (Liu and Kubli, 2003). Sex peptide receptor mutant females were of the genotype w^-*, Df(1R) SPR/FM7;+;+;+* (Yapici et al., 2008) and sex peptide control females were generated by crossing sex peptide receptor mutant females to *CS* males (*SPR* is a recessive allele). All flies used in this study were reared on fly food medium (recipe: agar (10 g/l), glucose (30 g/l), sucrose(15 g/l), yeast (35 g/l, Red Star active dry yeast, *S. cerevisiae*), cornmeal (15 g/l), wheat germ (10 g/l), soy flour (10 g/l), molasses (30 g/l), propionic acid (5 ml of 1M) and tegosept (2 g in 10 ml ethanol)) in an incubator (25 °C, 12:12 hour light-dark cycle (LD 12:12) with lights on at 09:00 (ZT 0)). Fly stocks were established by placing 20 males and 20 females into fly rearing bottles (polypropylene, 177 ml, filled with 45 ml fly food medium) and transferred into fresh bottles for egg-laying once or twice a week.

Mating arenas with partitioned food

Mating arenas were prepared by assembling 1 dextrose patch (1% agar, 2% dextrose) and 1 yeast patch (1% agar, 2% dextrose, 3.5% active dry yeast) embedded in agar in a 90 mm×15

mm arena (Fig 1a). Dextrose solution was made by suspending 1 g of agarose and 2 g dextrose in 100 mL of water, heating with frequent agitation, and boiling to 95 °C to completely dissolve agarose and dextrose on a magnetic stirrer with a heat plate. After cooling to 55°C, 18 mL of dextrose solution was transferred to a 90 mm × 15 mm plastic petri dish and the container was kept open until the agar hardened. Yeast solution was prepared as above with the addition of 3.5 g active dry yeast followed by boiling at 95 °C for 10 min to kill yeast. After cooling down, patches were generated by cutting the discs into equal 4 parts. A 35 mm×10 mm plastic petri dish was used to cut and remove the centre of each disc. A mating arena (Figure 1a) was created by transferring one sector of dextrose disc and one sector of yeast disc respectively to a new 90 mm×15 mm plastic petri dish (bottom diameter - 88 mm, height -12 mm, food height - 4 mm), arranging them in opposite direction and then pouring 9 ml 1% agarose solution (about 55°C) in between. The relative surface area of yeast (or dextrose) patch was calculated by dividing the area covered by yeast/dextrose ($(\pi 44^2 - \pi 17.5^2)/4$) by the total area (bottom area + wall area = $\pi 44^2 + 2\pi * 44 * 8$). The relative surface area of wall was calculated by dividing wall area ($2\pi * 44 * 8$) by the total area and the relative surface area for agar was calculated as the rest.

Acetic acid, peptone and acetic acid & peptone arenas were prepared with dextrose patches (1% agar, 2% dextrose) and acetic acid patches (1% agar, 2% dextrose, 1% acetic acid), peptone patches (1% agar, 2% dextrose, 3.5% peptone), acetic acid & peptone patches (1% agar, 2% dextrose, 3.5% peptone, 1% acetic acid) respectively. These patches were made in the same way as dextrose patches except that acetic acid was added to the dextrose solution or dextrose solution with peptone at about 40 °C to avoid evaporation. All patches were made 2-3 hours before experiments.

Two-yeast arenas were prepared with *C. boidinii* patches (1% agar, 2% dextrose, 3.5% *C. boidinii*) and *C. californica* patches (1% agar, 2% dextrose, 3.5% *C. californica*). *C. boidinii* and *C. californica* were kept on solid YPD medium (yeast extract 1%, peptone 2%, dextrose 2%, agar 2%) between experiments, harvested from fresh cultures produced by culturing a single colony overnight in 500 ml liquid YPD medium (same composition but without agar) in a 2 L glass flask with shaking (200 rpm) at 30 °C and by centrifugation in 50-ml tubes at 3000 rpm for 3 - 4 min. The supernatant was poured off and live yeast cells were diluted in an appropriate amount of dextrose solution to reach 3.5% yeast concentration and boiled till 95 °C for 10 min. *C. boidinii* and *C.californica* discs were cut into equal 8 parts and a 35 mm × 10 mm plastic petri dish was used to cut and remove the centre of each disc. Two-yeast mating arenas (Figure 5a) were generated by transferring one sector of *C. boidinii* disc and *C.californica* disc respectively to a new 90 mm × 15 mm plastic petri dish, arranging them in opposite direction and then pouring 12 ml 1% agarose solution (about 55°C) in between. The relative surface area of *C. boidinii* (or *C. californica*) patch was calculated by dividing the area covered by *C. boidinii* (or *C. californica*) ($(\pi 44^2 - \pi 17.5^2)/8$) by the total area (see above). The relative surface area of wall and agar was calculated in the same way as above.

Mating location assays

Virgin females and males were collected using CO_2 anaesthesia and aged in same-sex groups of 20-25 in food vials (25 × 95 mm) for 5-8 days. During an experiment, a virgin was transferred into one mating arena with the indicated food partitions and a male was placed later using a mouth pipette. Mating behaviour was quantified as described in Gorter and Billeter (2017). For every 2 min, a picture was captured with the webcam cameras (Logitech, 950270) and the monitoring software (Security Monitor Pro 5.16, DeskShare). All experiments began between Zeitgeber time (ZT) 6 and 7. After 24 h, pictures were analysed with image viewing software (FastStone Image Viewer, FastStone Soft). The location (region) of each mating was recorded. When the pair location changed during mating, the mating durations in each region were recorded and mating location was reported as that in which most time was spent. This occurred in less than 10% of the observations. When mating happened for the same duration at two locations, the region where mating started was used. This occurred in less than 3% of the observations.

For sex peptide experiments (Figure 4), 24 h before introducing females and males into arena, *CS* virgin females were mated either with *SP* null males or *SP* control males by keeping 10-15 pairs in one vial; males were removed after 1h. Twenty-three hours after mating, mated *CS* females were tested with *CS* males in the mating arenas for 24h. We used the same procedure for the sex peptide receptor experiment, except that either *SPR* mutant females or *SPR* control females were mated with *CS* males for the first mating and for rematings. We repeated these experiments when we tested *SP* mutant males and *SPR* mutant females 29 h before introducing females and males into the arena.

Behavioural tracking of location

Bowl-shaped arenas (Figure 2a) were printed with a 3D printer (Creality Ender 3 V2, Creality 3D) using PETG filaments (Material 4 Print, 1.75 mm, M4P 29600511121). The bottom of these arenas was covered with yeast, dextrose and agar pads as described above. The arenas were covered by a glass lid (110 × 110 mm) coated with Sigmacote (Sigma-Aldrich, SL2-100ML) to prevent flies from walking on the glass. Arenas were backlit with both white and red LEDs (LED StripXL, clear white (4000 - 4500 K), LED04009-1; red (640 - 660 nm), LED05004-1). Each bowl-shaped arena was placed under one Raspberry Pi camera (RASPBERRY PI, SC0261) at a resolution of 1920 × 1080 pixels and 15 fps for 12 hours in a photography box (Caruba, 2303765, 50 × 50 × 50 cm) in which 4 fans (Arctic Cooling PC Fan, 990365, 120 × 120 × 25 mm) were mounted to prevent condensation on glass covers of arenas. The device was kept in a temperature-controlled room set at 25 °C, 12:12 hour light-dark cycle (LD 12:12, lights on at 09:00 (ZT 0)). Arenas were used only once to prevent build-up of aggregation pheromones (Dumenil et al., 2016), but the glass lids were reused, after washing with ethanol twice and drying for 2-3 h after recording. Arenas were kept in the photography box 1 h before recording to maintain the same temperature as in the tracking box and avoid condensation in the glass lids. All experiments began at around Zeitgeber time (ZT) 6 and lasted for 12h. To measure the locations of single mated females and mated males, a pair of virgin female and male was transferred into one arena for mating for 1h and then

either the female or the male was removed. To measure the location of single virgin females and pairs of virgin females and males, a virgin female and a female-male couple were transferred into one mating arena respectively using a mouth pipette. The relative surface area of the yeast (or dextrose) patch was calculated by dividing the yeast/dextrose area ($(\pi 44^2 - \pi 17.5^2)/4$) by the total area (bottom area + wall area = $\pi 44^2 + (\pi * 52 * 52 * \sqrt{2} - \pi * 44 * 44 * \sqrt{2})$). The relative surface area of wall was calculated by dividing the wall area ($\pi * 52 * 52 * \sqrt{2} - \pi * 44 * 44 * \sqrt{2}$) by the total area; the relative surface area of agar was calculated as the rest.

After video recording, videos were converted from h264 to mp4 and analysed with python-based tracking software – TRex (Walter and Couzin, 2021). Converted videos were cropped in TRex to keep only the targeted dish in view and segmented to extract all the potential moving targets from their background. After cropping and segmentation, a background image and a non-proprietary video format (PV) file storing only the coordinates of potential targets were saved separately for the following tracking. After loading the PV file and setting up the tracking parameters like the blob_size_range and number of individuals to be tracked, individuals were tracked in TRex automatically and can be manually corrected for their identities when females and males overlap during copulations. Tracking data were saved to independent data-containers (NPZ) using the save function in the menu of TRex and were visualized and calculated by Matlab script (appendix 1).

Statistical analysis

Preference for mating on a specific substrate was tested by comparing the percentage of mating occurring on that substrate in 24 h with the relative surface area of that substrate using chi-square test in R (v 4.0.3, 2020; R Development Core Team, 2020). We used the same protocol to test the preference of locating on a specific substrate. But we calculated the percentage of time individuals spent on that substrate for each hour. The effects of time since entering the arenas, light conditions and mating history on the likelihood of mating on yeast were tested with the generalized linear mixed models using the lme4 package (Bates et al., 2015) in R. Time since entering the arenas, light conditions and mating history were included as fixed effects, and the date of experiments and the identity of the containers in which flies were exposed to different substrates were included as independent random effects. Whether *SP* and *SPR* are involved in mediating mating location differences between virgin and mated females and the effects of yeast components (i.e., acetic acid, peptone or the combination of both) on the probability of remating on that yeast components were also tested with the generalized linear mixed models using fly genotype (yeast components), light conditions and interactions between them as fixed effects, and the date of experiments, time since entering the arenas and the identity of the containers as independent random effects. Mating location preference between *C. boidinii* and *C. californica* was tested with the generalized linear mixed models by including time since entering the arenas and light conditions as the fixed effects and the date of experiments and the identity of the containers as independent random effects. When fixed effect parameter has several levels, we performed multiple comparisons

using posthoc Tukey test (emmeans package, Lenth, 2020) to identify the differences between these different levels.

Results

1. *Drosophila* prefers (re)mating on yeast patches. We monitored the mating location of pairs of freely interacting males and females in a mating arena, partitioned in different sectors containing either agar only, dextrose mixed with agar (dextrose patch) or yeast mixed with dextrose and agar (yeast patch) (Figure 1a) for 24 h. We found that 84% of the time, the first mating of these pairs (virginal mating) happened on the wall of the arena, while most subsequent matings (re-mating, 56%, 84% and 85% for 2^{nd}, 3^{rd} and 4^{th} mating respectively) took place on the yeast patch (Figure 1b, Table S1). Preference for remating on the yeast patch was not specific to lab strain *CS*, but found in all *D. melanogaster* strains examined, namely *OR*, w^{1118}, *I16* and *N01* (Figure 1c, Table S1), suggesting it is a general feature of this species. In contrast, three other *Drosophila* species (*simulans*, *suzukii* and *yakuba)* mated only once in 24 h and mated on the yeast patch directly (Figure 1d, Table S1). Mating rate was not the only determining factor in mating location, since *D. melanogaster* strain *N01* seldomly remated, yet still did not mate on the yeast patch for the virginal mating (Figure 1c). Taken together, these data show that *Drosophila* generally mates on yeast substrates, but in *D. melanogaster* mating status is a critical determinant with virgin females avoiding mating on yeast substrates.

In *D. melanogaster*, the proportion of pairs mating on the yeast patch increased with the number of matings (Figure 1b). There are at least two, not mutually exclusive, explanations for this transition. First, the probability to mate on yeast may increase with the time elapsed since entering the arena, due to habituation to the arena or a gradual post-mating response transition from virgin to mated in females. Second, mating location may be influenced by light conditions since flies entered the arena in lights-on conditions but experienced a switch to darkness 5 hours later (since arenas were exposed to a 12:12 LD cycle). To explore how time and light conditions affect the likelihood of mating on yeast, we investigated mating location under both a light cycle of 12L:12D (Figure 1e, data from Figure 1b) and in constant darkness (24D) (Figure 1f). We found that the probability of mating on the yeast patch increased with time ($\chi 2 = 12.936$, Df = 1, $P<0.001$; Figure 1e, Table S2) and was higher in the dark in both light conditions ($\chi 2 = 17.110$, Df = 1, $P<0.001$; Figure 1 e, f, Table S2). We conclude that female mating status, time since the entry of females and males into the arena and light condition all influence mating location.

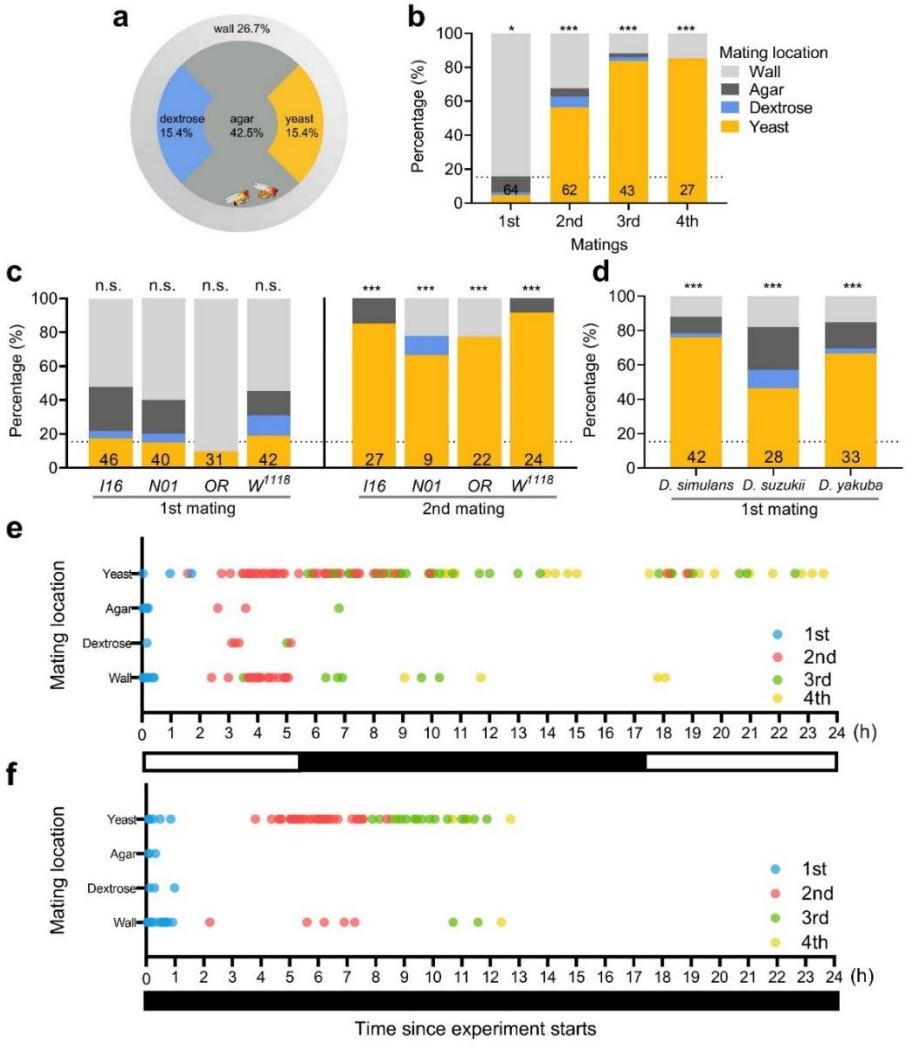

Figure 1 | Mating location of *Drosophila* in 24h. (a) Schematic representation of the mating location assay. A mating arena was divided in four sectors: wall (light grey), agar only (dark grey), dextrose mixed with agar (dextrose patch, denoted as dextrose, blue) and yeast mixed with dextrose and agar (yeast patch, denoted as yeast, orange). Numbers alongside text are the relative surface area of each sector. (b) Percentage of matings occur on four sectors for the observed 4 matings (1st, 2nd, 3rd, 4th) in *CS*. Numbers listed at the bottom of each bar are the number of replicates. (c) Percentage of matings occur on four areas in four other *D. melanogaster* strains: *I16, N01, OR, w1118* for the observed 2 matings (1st and 2nd mating). (d) Percentage of matings occurring on four sectors in three other *Drosophila* species: *D. simulans, D. suzukii* and *D. yakuba* for the observed 1st mating. (e, f) Mating location (four

sectors indicated on y axis) of *CS* under a light cycle of 12L: 12D (e, data from b) and in constant darkness (24D, f) for the observed 4 matings (1st, blue; 2nd, red; 3rd, green; 4th, yellow). The reference line is the relative surface area of the yeast patch. Data were analysed using chi-square test (b, c, d) and binomial generalized linear mixed model (e & f). ** $p<0.01$, *** $p<0.001$. For detailed statistical analysis, see Supplementary Table 1 & 2.

2. Sex peptide and sex peptide receptor mediate mating location differences between virgin and mated females. To assess the potential impact of mating location preference on assortative mating, we need to understand what causes the behavioural difference between virgin and mated females. The sex peptide (*SP*) produced in *D. melanogaster* male accessory glands and transmitted to females during copulation, together with the sex peptide receptor (*SPR*) that relays sex peptide-induced signals, generate a suite of post-mating responses in females (e.g., elevated egg-laying and food intake, reduced receptivity) (Liu and Kubli, 2003; Yapici et al., 2008; Kubli and Bopp, 2012). Both *SP* and *SPR* contribute to dietary preference for yeast in females after mating (Ribeiro and Dickson, 2010), suggesting they may also be involved in mediating the different mating locations of virgin and mated females. We tested this hypothesis using *SP* mutant males and *SPR* mutant females. These mutants were tested after 24 h since it takes more than 12 h for females lacking *SPR* or *SP* to behave like virgin females (Liu and Kubli, 2003). We also tested these mutants under different light conditions and times elapsed since entering the arena as we found these two factors influenced mating location in the above experiments. We found that wild-type females that mated with *SP* null males behaved like virgin females: they mated fast and preferred to mate on the arena walls, under both light and dark conditions (Figure 2 light: a1, a2; dark: b1, b2, Table S1 & S2). In contrast, wild-type females that mated with *SP* control males mated late and preferred to mate on the yeast patch (Figure 2 light: a1, a2; dark: b1, b2, Table S1 & S2). Concordant with the pattern observed in *SP* mutants, mated *SPR* mutant females behaved like virgins, mating fast and preferring to mate on the arena walls under both light and dark conditions (Figure 2 light: c1, c2; dark: d1, d2, Table S1 & S2), while mated *SPR* control females mated late, mainly mated in dark phase and preferred to mate on the yeast patch (Figure 2 c1, c2; d1, d2, Table S1 & S2). In dark conditions, mated *SPR* mutant females remated later and more on the yeast patch compared with light conditions, but these females still preferred to mate on the arena walls (Figure 2 light: c1, c2; dark: d1, d2, Table S1 & S2). This indicates that sex peptide modulates mating location preference between virgin and mated females. Together, we conclude that the mating location differences between virgin and mated females are caused by *SP* and *SPR*.

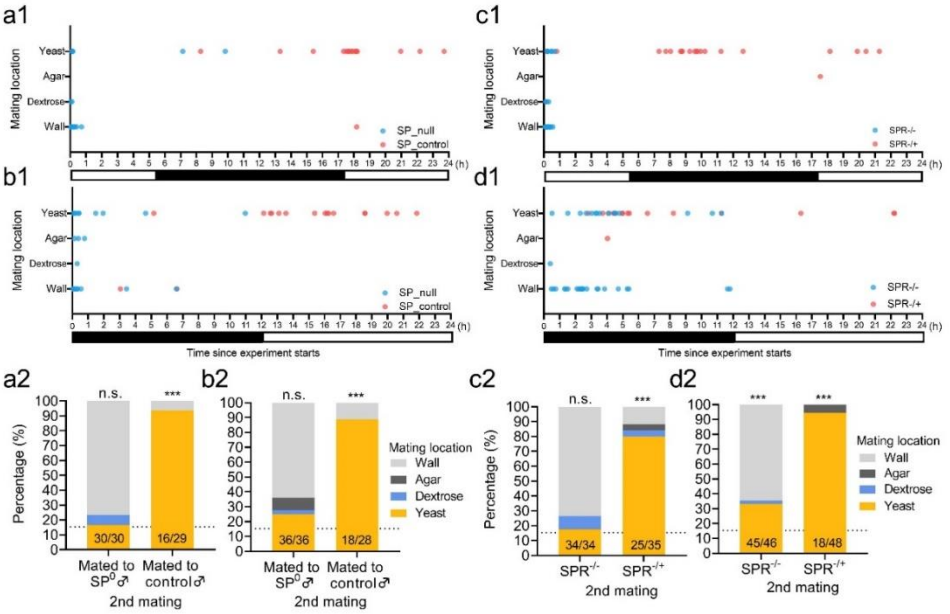

Figure 2 | Mating location of *SP* and *SPR* mutants during the 2nd mating under different light conditions and times elapsed since entering the arena. (a1, a2) Mating location of *CS* females mated with either *SP* null males or *SP* control males 24h before test (introducing females and males into the mating arenas). (b1, b2) Mating location of *CS* females mated with either *SP* null males or *SP* control males 29 h before test. (c1, c2) Mating location of *SPR* mutant females and control females mated 24h before test. (d1, d2) Mating location of *SPR* mutant females and control females mated 29 h before test. The reference line is the relative surface area of the yeast patch. Numbers listed at the bottom of each bar are the number of replicates tested (left of the slash) and the number of replicates mated (right of the slash). Data were analysed using chi-square test (a2, b2, c2, d2) and binomial generalized linear mixed model (a2 & b2, c2 & d2). ** $p<0.01$, *** $p<0.001$. For detailed statistical analysis, see Supplementary Table 1 & 2.

3. Flies locate on yeast at night. Our observation that *D. melanogaster* pairs tend to mate on arena walls for virginal mating and yeast substrate for rematings, combined with the previous finding that mated females rather than virgin females and males are strongly attracted to yeast after yeast deprivation (Ribeiro and Dickson, 2010), leads us to investigate where individuals locate and whether individuals locate on different substrates based on their mating statuses and sexes. To explore this, we used continuous automated video tracking (Figure 3a, b, c, d) to determine the location of individual virgin females, mated females and males. We found that individual virgin and mated females located on the arena walls during the day, but moved onto yeast patch and remained there at night (Figure 3e). The pattern of shifting from wall to yeast at night was also observed in individual males, but during the day

males were more often located on the yeast patch than females (Figure 3e, Table S2). These results suggest that light conditions and/or the circadian clock may affect individuals' location. To explore this, we tracked single virgin and mated females as well as males under constant dark conditions. We found that both individual virgin and mated females moved onto the yeast patch and remained on yeast during the subjective night, but they spent more time on yeast during the subjective day in 12D than they did in the 6L:6D light condition (Figure 3f, Table S2). The shift to yeast at night was absent in individual males as they spent similar amounts of time on yeast during the subjective day and during the subjective night, and less time on yeast during the subjective night in 12D compared with 6L:6D light conditions. These results demonstrate that when alone, individual females of different mating statuses distribute themselves similarly, but sexes differ in how much time they spend on the arena walls and on the yeast patch. Together, we conclude that the individuals' location is thus not determined by mating status, but by sex and light conditions and/or intrinsic circadian rhythm.

Male-female pairs behave differently than individual flies since the presence of another sex triggers courtship (Fujii et al., 2007). Therefore, it is important to track individuals in pairs with another sex to explore where sexes locate and whether the presence of another sex affects individuals' location. We thus continuously tracked the location of pairs of females and males for 12 h under a 6L:6D light cycle. We found that mated females in pairs with males were mainly located on the wall before they became receptive for remating, then moved onto the yeast patch during the light phase and remained on the yeast patch during the dark phase (Figure 3g). Males in pairs with females exhibited a similar pattern as females (Figure 3g). Since we found that individuals' location was influenced by light condition and/or intrinsic circadian rhythm in the above experiments, we thus also tracked the location of pairs of females and males under constant dark conditions for 12 h. We found that males in pairs with females mainly stayed on yeast patch during the subjective day and both sexes shifted to yeast patch during subjective night in constant dark condition (Figure 3h, Table S2). These results demonstrate that flies either alone or in pair with another sex only mainly locate on yeast at night. By comparing individuals alone and individuals in pairs with another sex, we found that the presence of males increased the time females spent on the yeast patch during the light phase compared with individual females, while males in pairs with females spent similar amounts of time on yeast as when they were alone (Figure 3i). On the other hand, the presence of females increased the time males spent on the yeast patch under constant dark conditions (Figure 3j). This indicates that the presence of another sex increases individuals' tendencies to spend time on the yeast patch. Together, we conclude: flies either alone or in pairs with another sex locate on yeast at night, but individuals' tendencies to be on the yeast during the day depend on several variables including sex, light conditions and presence of another sex.

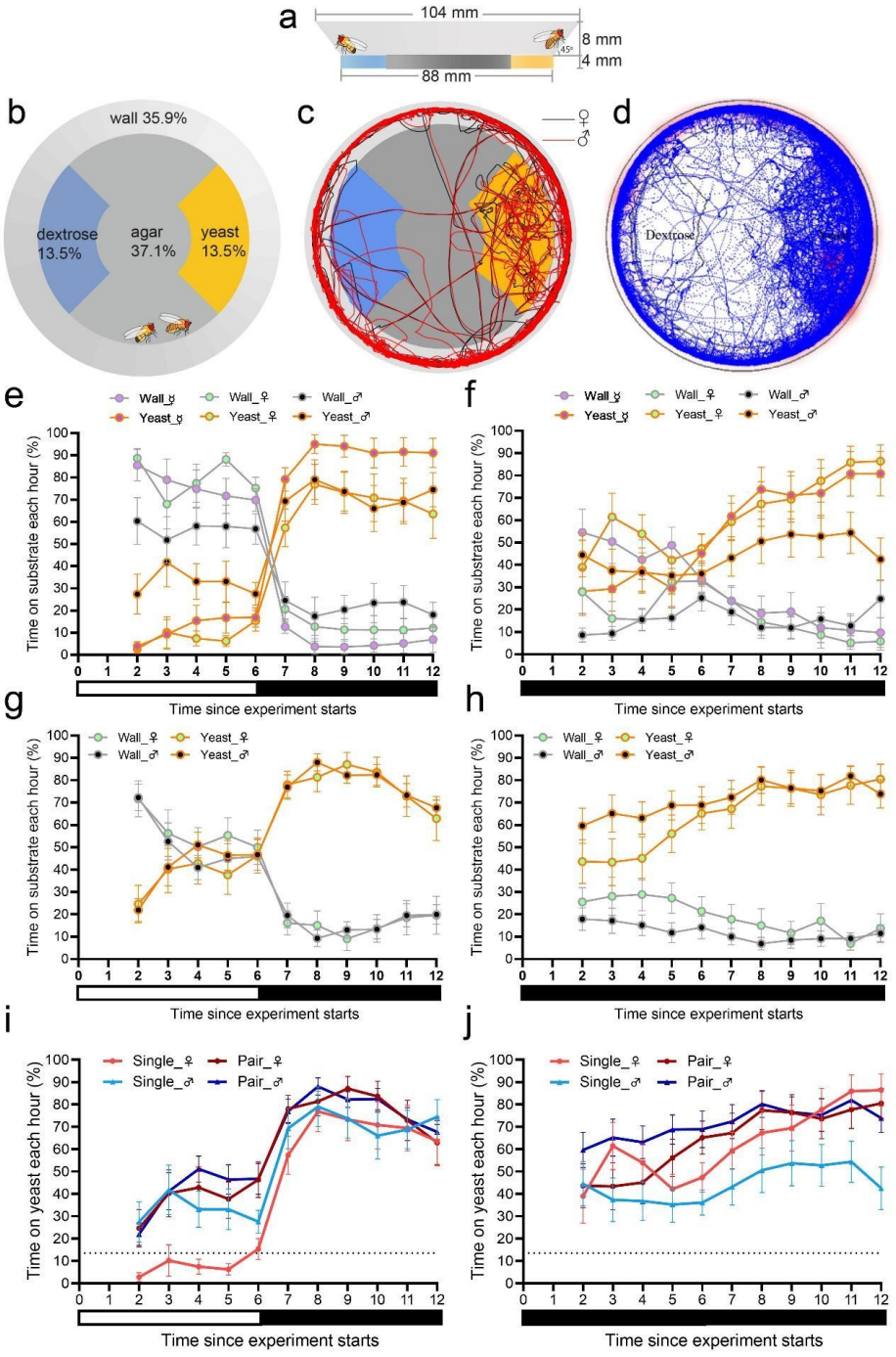
a
104 mm
8 mm
4 mm
88 mm
45°
b
wall 35.9%
dextrose 13.5%
agar 37.1%
yeast 13.5%
c
♀
♂
d
Dextrose
e
Wall_☿
Yeast_☿
Wall_♀
Yeast_♀
Wall_♂
Yeast_♂
Time on substrate each hour (%)
Time since experiment starts
f
Wall_☿
Yeast_☿
Wall_♀
Yeast_♀
Wall_♂
Yeast_♂
Time on substrate each hour (%)
Time since experiment starts
g
Wall_♀
Yeast_♀
Wall_♂
Yeast_♂
Time on substrate each hour (%)
Time since experiment starts
h
Wall_♀
Yeast_♀
Wall_♂
Yeast_♂
Time on substrate each hour (%)
Time since experiment starts
i
Single_♀
Pair_♀
Single_♂
Pair_♂
Time on yeast each hour (%)
Time since experiment starts
j
Single_♀
Pair_♀
Single_♂
Pair_♂
Time on yeast each hour (%)
Time since experiment starts

Figure 3 | (a, b) Schematic representation of the bowl-shaped arena for behavioural tracking: a) side view; b) top view. (c, d) Illustration of behavioural tracking for one hour: c) trajectory of a pair of flies- female (♀) and male (♂), d) heatmap of trajectories of 14 females (in blue). (e, f) The percentage of time individual virgin and mated females as well as males spent on wall and yeast for each hour under light condition 6L: 6D (e) and 12D (f). ☿ – virgin female, ♀ – mated female, ♂- mated male. The number of replicates for each fly group at each time point ranges from 11 to 16. (g, h) The percentage of time male-female couples spent on wall and yeast for each hour under light condition 6L: 6D (g) and 12D (h). ♀ – female, ♂- male. The number of replicates at each time point ranges from 12 to 15. (i, j) Comparison between the percentage of time females (males) in pairs and females (males) alone spent on yeast for each hour under light condition 6L: 6D (i) and12D (j). The reference line is the relative surface area of the yeast patch. Data were analysed using binomial generalized linear mixed models (e & f, g & h). For detailed statistical analysis, see Supplementary Table 2.

4. Combination of acetic acid and peptone attracts flies to remate on yeast. Given that flies are attracted to yeast for (re)mating, we next asked what cues attract the flies. In a previous study (Gorter et al., 2016), the combination of acetic acid (a yeast-derived volatile) and peptone (a nutritional protein) was found to modulate sexual receptivity of *D. melanogaster*, suggesting that this combination of compounds may also attract flies to mate on yeast. To test this, we replaced yeast by either acetic acid, peptone or the combination of both. We found that replacing yeast by either acetic acid or peptone alone did not generate a preference for remating on either of them (Figure 4 a, b, d). But their combination induced strong preferences for remating on this substrate (Figure 4 c, d, Table S2). We also found that flies were more attracted to remate on this combination of both at night than during the day ($\chi 2 = 13.227$, Df = 1, $P<0.001$), confirming that darkness increases the tendency to remate on the combination of acetic acid and peptone (Figure 4c, Table S2).

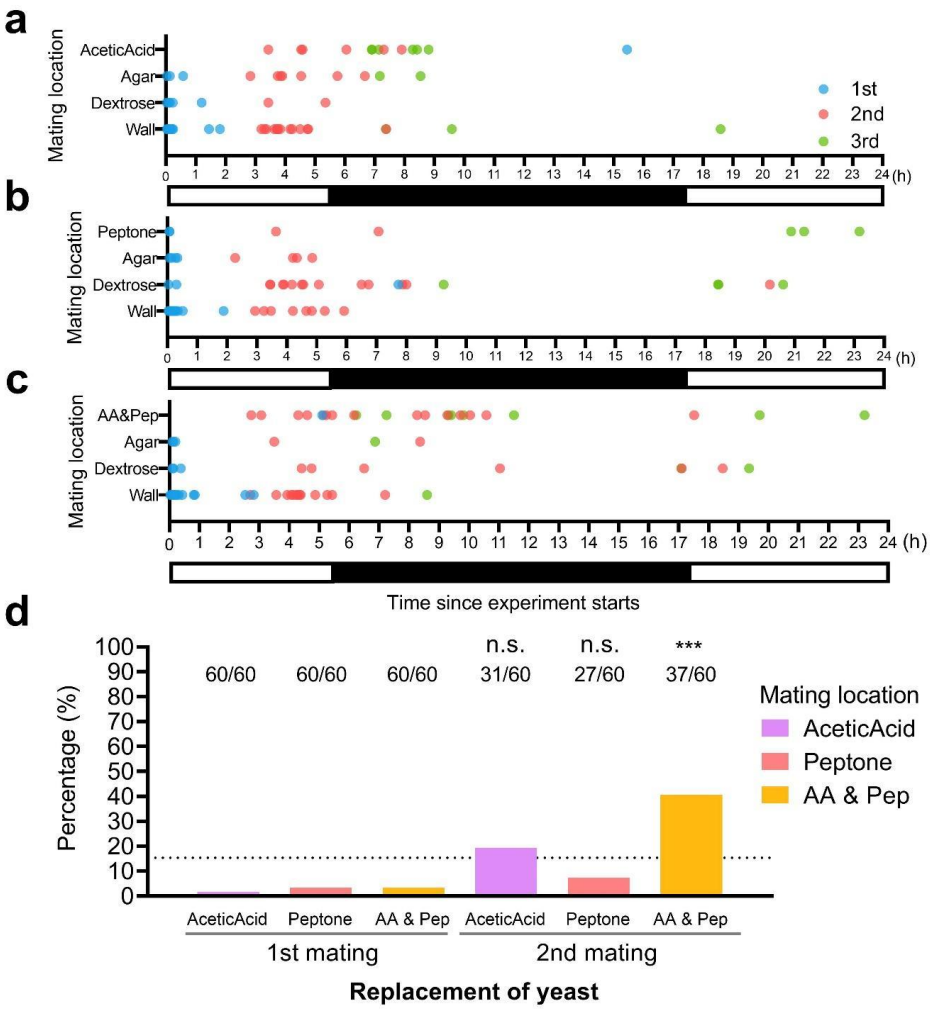

Figure 4 | Mating location of *D. melanogaster* when yeast was replaced by acetic acid (a), peptone (b) or the combination of acetic acid and peptone (c) for the observed 3 matings (1st, blue; 2nd, red; 3rd, green) in 24h. Four sectors were indicated on y axis. (d) Percentage of matings occurring on acetic acid, peptone and combination of acetic acid and peptone for the observed 1st and 2nd mating. Numbers listed on top of each bar are the number of replicates. AA & Pep – Acetic acid & peptone. The reference line is the relative surface area of the yeast patch. Numbers listed on top of each bar are the number of replicates tested (left of the slash) and the number of replicates mated (right of the slash). Data were analysed using chi square test (d) and binomial generalized linear mixed model. ** $p<0.01$, *** $p<0.001$, n.s.-not significant. For detailed statistical analysis, see Supplementary Table 1 & 2.

5. *D. melanogaster* prefers *C. boidinii* over *C. californica* for remating. Flies can discriminate between different yeast species based on yeast odours (Scheidler et al., 2015) and prefer to feed on particular yeast species rather than others (Cooper, 1960). Our finding that flies move to yeast for (re)mating, combined with the above-mentioned feeding preference for certain yeast species, leads us to hypothesize that flies will also show preferences for particular yeast species for (re)mating. We tested our hypothesis by exposing several wild-type fly strains to two different yeast species, i.e., *C. boidinii* and *C. californica* (Figure 5a). All the *melanogaster* strains we tested preferred *C. boidinii* over *C. californica* for remating (Figure 5 c, d), but the time since the entry of females and males into the arena affected yeast species preference (χ^2 = 5.8339, Df = 1, P = 0.016). For rematings, *CS* initially preferred *C. californica*, but switched to *C. boidinii* approximately 7 h after the entry for subsequent matings. This suggests that flies may not stay on one substrate when multiple substrates are available, possibly limiting the opportunity for spatial segregation and assortative mating. We found that females preferred to oviposit on the yeast patch where their most recent mating took place, i.e., *C. boidinii* (Figure 5e), suggesting that oviposition location may be spatially coupled with mating location: flies lay their eggs where they prefer to mate. Together, these results suggest that there is scope for yeast-mediated assortative mating in *D. melanogaster*, but it is influenced by many factors (fly strain, yeast species, temporal dynamics of (re)mating).

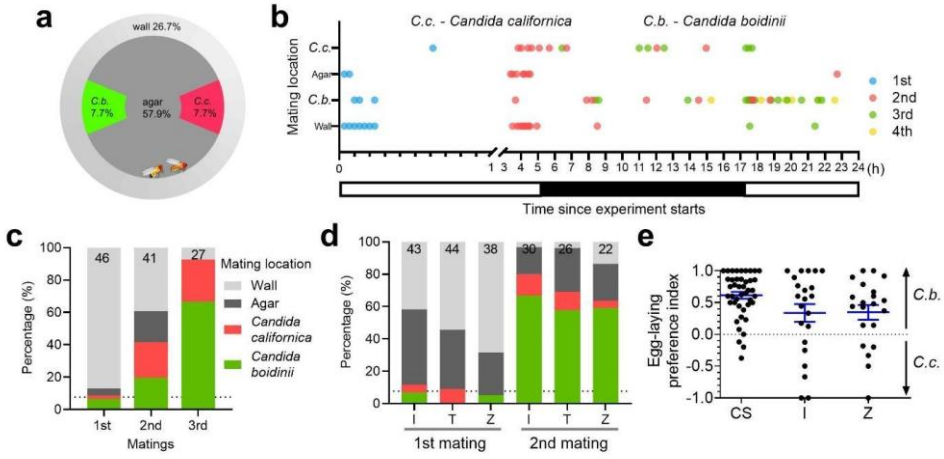

Figure 5 | Mating location preference of *D. melanogaster* for *Candida boidinii* vs. *Candida californica*. (a) Schematic representation of the mating location preference assay. A mating arena was divided by four sectors: wall (light grey), agar only (dark grey), *C. boidinii* mixed with dextrose and agar (*C. boidinii* patch, denoted as *C.b.*, green) and *C. californica* mixed with dextrose and agar (*C. californica* patch, denoted as *C.c.*, red). Numbers alongside text are the actual ratios of each sector. (b) Mating location of *CS* with two-yeast choice for the observed 4 matings (1st, blue; 2nd, red; 3rd, green; 4th, yellow). Four sectors were indicated on

y axis. (c) Percentage of matings occur on four sectors for the observed 3 matings (1st, 2nd and 3rd) in *CS*. Numbers listed at the top of each bar are the number of replicates. (d) Percentage of matings occur on four sectors for the observed 2 matings (1st and 2nd) in strains of *I16*, *T01* and *ZW*. (e) Egg-laying preference of strains *CS*, *I16* and *ZW*. The reference line is the relative surface area of the *C. boidinii* patch. Data were analysed using binomial generalized linear mixed model (c). For detailed statistical analysis, see Supplementary Table 2.

Discussion

In this study, we explored the sensory and behavioural mechanisms underlying mating location preference in *Drosophila*. We experimentally demonstrate that *Drosophila* generally mates on yeast, but in *D. melanogaster* mating status is a critical determinant. The underlying mechanism of the spatial coupling of food and mates in *D. melanogaster* involves yeast-derived chemical cues (the combination of acetic acid and protein) and the sex peptide received and sensed by females. Multiple factors including mating status, light condition and time influence mating location. We also find that mated females prefer to remate (and oviposit) on one yeast species rather than another, and this preference changes with time. Together, our study suggests that some level of assortative mating may result from the preference for mating on yeast, but the strength of such assortative mating will depend on several variables including the presence of multiple yeast species and timing of (re)mating with respect to light cycle.

We find that virgin *D. melanogaster* females prefer to mate on the walls of the experimental arenas. The behaviour of the virgin females might be explained by centrophobism, i.e., avoiding the centre after entering an open space (Besson and Martin, 2005; Soibam et al., 2012; Mohammad et al., 2016), since virginal mating happens quickly after the introduction into the arena. Wall-following behaviour in *Drosophila* has been interpreted as anxiety (Mohammad et al., 2016) or a preference for the arena boundary (Soibam et al., 2012). In semi-natural conditions, newly eclosed virgin females – typically in the early morning - leave their pupae, walk away from the foraging substrate and only come back during the dark phase (Stamps et al., 2005), possibly indicating that virgin females stay away from food to avoid male harassment or predation. *D. melanogaster* has been observed mating on fruits in the field (Markow, 1988; Soto-Yeber et al., 2018) and our study expands on these field observations by revealing the difference in mating location between virgin and mated females. Our study which employs a standardized choice between alternative substrates enables us to experimentally measure mating location and minimize the influence of uncontrolled environmental factors.

We find that sex, social environment, circadian rhythm and light conditions together modulate how individuals move between different substrates. Sex differences in fly presence on different substrates during the day was also observed in field studies (Markow, 1988) and in semi-natural conditions (2.6 × 2.6 × 3.6 m room with food, perches and hiding places for

free-moving flies; Stamps et al., 2005). Males spend more time at food resources than non-food substrate (e.g., leaves) in the semi-natural condition. This may be because of mating opportunities, as receptive females are found predominantly on food resources and food also increases female receptivity (Markow, 1988; Gorter et al., 2016). Indeed, we find that males maintained similar locomotor activity to females and followed them everywhere they went. Females generally moved more often, in line with the field observations that females are often harassed by males around food resources (Wertheim et al., 2006; Soto-Yeber et al., 2018). Moreover, females spent more time on yeast in the presence of males than when alone. Nevertheless, both individual flies (virgin female, mated female and mated males) and male-female couples spent most time on yeast during the night, as well as in darkness during the subjective day, corresponding with previous findings that circadian rhythm as well as light condition influence *Drosophila* feeding (Xu et al., 2008) and mating behaviour (Fujii et al., 2007; Shahandeh et al., 2020) and suggesting that the factors controlling these behaviours also control their coupling in space. We found that individual flies of all sexes spend time on yeast at night. However, they may gain different benefits from doing so. We hypothesize that individual virgin females, mated females and mated males may go to yeast for food, oviposition and mates, respectively. We infer this from observing that virgin females remained on the yeast patch without moving, mated females moved a bit in the yeast patch, and mated males moved very often and all over the yeast patch (Figure S1). However, more detailed behavioural observations involving the quantification of feeding events are required to test these hypotheses.

Notably, mating can also occur at locations separate from feeding substrates. These locations, where there are no food resources but aggregated males and visiting females, are termed leks (Shelly and Whittier, 1997). In nature, flies of the families Tephritidae and Drosophilidae congregate at leks for mating (Markow, 1988; Shelly and Whittier, 1997; Warburg and Yuval, 1997; Shelly, 2018). For example, the above-mentioned Mediterranean fruit fly, a ubiquitous fruit pest, feeds on a variety of fruits but mating takes place in the canopies of pitanga tree (*Eugenia uniflora*) (Warburg and Yuval, 1997). The cactophilic fly *Drosophila nigrospiracula* (Diptera: Drosophilidae), which feeds on rotting cacti (*Camegia gigantea*), mates on a non-resource-based male territory (Markow, 1988). The picture-wing clade of Hawaiian *Drosophila*, endemic to the Hawaiian Islands, mainly feeds on decaying stems and sap exudates of native trees (Montgomery, 1975), but forms leks and mates on the leaves of trees (Bell and Kipp, 1994; Shelly, 2018). Thus, lekking behaviour occurs in a variety of fly species. Yet, it is unknown whether species that form leks and mate away from their food resources are less likely to evolve food-associated reproductive isolation (i.e., lower rates of host race formation). We propose that comparative studies between lekking and non-lekking species may provide a good opportunity to understand the evolution of food-associated reproductive isolation.

Mating with more than one partner is the general rule rather than the exception in the animal kingdom (Kvarnemo and Simmons, 2013). By mating with multiple males, females receive a wide variety of benefits including removing the risk of remaining unmated, security of

fertility, increased egg production, better offspring fitness with higher genetic compatibility and genetic diversity, more paternal care and protection for themselves and their offspring (Kvarnemo and Simmons, 2013; Kohlmeier et al., 2021). We observe that *D. melanogaster* is different from sibling species in which virginal mating occurs relatively late, since it mates fast and away from yeast for virginal mating, suggesting that closely related *Drosophila* species may have different strategies regarding mating location. Following multiple mating in *D. melanogaster*, the last male sires most of the offspring (Morrow et al., 2005). Here, we find that *D. melanogaster* females show strong remating and oviposition preferences for specific yeast species. Consequently, most offspring will develop on their parents' preferred yeast species, generating opportunities for yeast-mediated spatial isolation between offspring derived from parents with different preferences. Yet, the preference of remating on a certain yeast is time-dependent. These results suggest that studies of food-mediated assortative mating should consider the entire mating trajectories of individual flies, and extended time intervals (the duration for at least two matings).

We observed an increased probability of mating on yeast in dark conditions. Darkness may hamper males' ability to detect females, which is based on visual perception (Agrawal et al., 2014; Ribeiro et al., 2018). Darkness may thereby delay mating and increase the time elapsed since the pair entered the arena, increasing the probability of mating on the yeast patch. This potential role of circadian rhythm and light conditions in fly (mating) location introduces another temporal dimension in food-mediated assortment. Our study does not provide direct evidence for assortative mating associated with yeast choice, since all *melanogaster* strains we tested prefer *C. biodinii*. Yet, our observations reveal the possibility of assortative mating based on yeast preference through the spatial coupling of mating and food, and opens new avenues for future research. Our study might also contribute to improving behaviour-based management programmes for pest control (e.g., using yeast to attract flies to insecticide formulations (Rehermann et al., 2022)), since we show when these flies are at what places and how males and females interact and mate on food substrates.

Supplementary information

Table S1 Comparisons of proportions of mating occurring on four sectors (agar, dextrose, wall and yeast) to the respective theoretical proportions (42.5%, 15.4%, 26.7%, 15.4%) using chi-square test. Significant effects ($p<0.05$) are indicated in asterisks. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure panel	Result	Posthoc	Standardize	Significance
Fig 1b-1st mating	$\chi2 = 108.98$, df = 3, p < 2.2e-16	agar	-5.36	****
		dextrose	-3.07	**
		wall	10.43	****
		yeast	-2.37	*
Fig 1b-2nd mating	$\chi2 = 92.48$, df = 3, p < 2.2e-16	agar	-6	****
		dextrose	-1.95	.
		wall	0.99	n.s.
		yeast	8.96	****
Fig 1b-3rd mating	$\chi2 = 155.09$, df = 3, p < 2.2e-16	agar	-5.33	****
		dextrose	-2.38	*
		wall	-2.23	*
		yeast	12.41	****
Fig 1b-4th mating	$\chi2 = 102.44$, df = 3, p < 2.2e-16	agar	-4.47	****
		dextrose	-2.22	*
		wall	-1.4	n.s.
		yeast	10.05	****
Fig 1c-*I16*-1st	$\chi2 = 17.863$, df = 3, p = 0.0004695	agar	-2.25	*
		dextrose	-2.08	*
		wall	3.91	****
		yeast	0.37	n.s.
Fig 1c-*N01*-1st	$\chi2 = 24.191$, df = 3, p = 2.279e-05	agar	-2.88	**
		dextrose	-1.82	.
		wall	4.76	****
		yeast	-0.07	n.s.
Fig 1c-*OR*-1st	$\chi2 = 65.606$, df = 3, p = 3.722e-14	agar	-4.79	****
		dextrose	-2.38	*
		wall	8.01	****
		yeast	-0.88	n.s.
Fig 1c-W^{1118}-1st	$\chi2 = 20.95$, df = 3, p = 0.0001078	agar	-3.7	***
		dextrose	-0.63	n.s.
		wall	4.11	****
		yeast	0.65	n.s.
Fig 1c-*I16*-2nd	$\chi2 = 101.62$, df = 3, p < 2.2e-16	agar	-2.91	**
		dextrose	-2.22	*
		wall	-3.14	**
		yeast	10.05	****
Fig 1c-*N01*-2nd	$\chi2 = 19.36$, df = 3, p = 0.0002303	agar	-2.58	**
		dextrose	-0.36	n.s.
		wall	-0.3	n.s.
		yeast	4.26	****
Fig1c-*OR*-2nd	$\chi2 = 67.557$, df = 3, p = 1.423e-14	agar	-4.03	****
		dextrose	-2	*
		wall	-0.42	n.s.

		yeast	8.04	****
Fig 1c-W^{1118}-2nd	$\chi2$ = 107.34, df = 3, p < 2.2e-16	agar	-3.39	***
		dextrose	-2.09	*
		wall	-2.96	**
		yeast	10.35	****
Fig 1d-*D. simulans*	$\chi2$ = 119.6, df = 3, p < 2.2e-16	agar	-4.32	****
		dextrose	-2.34	*
		wall	-2.17	*
		yeast	10.91	****
Fig 1d-*D. suzukii*	$\chi2$ = 20.742, df = 3, p = 0.00011	agar	-1.87	.
		dextrose	-0.69	n.s.
		wall	-1.06	n.s.
		yeast	4.55	****
Fig 1d-*D. yakuba*	$\chi2$ = 67.055, df = 3, p = 1.823e-14	agar	-3.18	**
		dextrose	-1.97	*
		wall	-1.5	n.s.
		yeast	8.16	****
Fig 2 b2 - SP null	$\chi2$ = 42.32, df = 3, p = 3.432e-09	agar	-4.71	****
		dextrose	-1.33	n.s.
		wall	6.19	****
		yeast	0.19	n.s.
Fig 2 b2 - SP	$\chi2$ = 75.549, df = 3, p = 2.763e-16	agar	-3.44	***
		dextrose	-1.71	.
		wall	-1.85	.
		yeast	8.68	****
Fig 2 b2 - SP null	$\chi2$ = 34.414, df = 3, p = 1.62e-07	agar	-4.15	****
		dextrose	-2.1	*
		wall	5.04	****
		yeast	1.6	n.s.
Fig 2 b2 - SP	$\chi2$ = 75.184, df = 3, p = 3.308e-16	agar	-3.65	***
		dextrose	-1.81	.
		wall	-1.5	n.s.
		yeast	8.64	****
Fig 2 c2 - $SPR^{-/-}$	$\chi2$ = 43.442, df = 3, p = 1.983e-09	agar	-5.01	****
		dextrose	-1.06	n.s.
		wall	6.17	****
		yeast	0.36	n.s.
Fig 2 c2 - $SPR^{-/+}$	$\chi2$ = 80.598, df = 3, p < 2.2e-16	agar	-3.89	***
		dextrose	-1.58	n.s.
		wall	-1.66	.
		yeast	8.95	****
Fig 2 d2 - $SPR^{-/-}$	$\chi2$ = 57.608, df = 3, p = 1.906e-12	agar	-5.77	****
		dextrose	-2.45	*
		wall	5.72	****
		yeast	3.33	***
Fig 2 d2 - $SPR^{-/+}$	$\chi2$ = 86.388, df = 3, p < 2.2e-16	agar	-3.17	**
		dextrose	-1.81	.
		wall	-2.56	*
		yeast	9.29	****

Table S2 Analyses of the effects of different factors on the probability of mating on yeast using binomial generalized linear mixed models. Significant effects (P<0.05) are shown in bold.

Figure	Model	Factors	Contrasts	χ2	Df	Z	P
1 e & f	MatingLocation~ Light+Time+MatingHistory+(1|Date)+(1|Dish)	**Light**		17.110	1		<0.00
		Time		12.972	1		<0.00
		MatingHisto		26.750	3		<0.00
			1st - 2nd			-	0.001
			1st - 3rd			-	0.485
			1st - 4th			0.172	0.998
			2nd - 3rd			1.669	0.340
			2nd - 4th			2.213	0.120
			3rd - 4th			1.335	0.540
			Dark - light			4.136	<.000
2 a2 &	MLocation ~ Genotype+Light +time+(1|Dish)+	Genotype		0.170	1		0.680
		Light		1.787	1		0.181
		Time		11.072	1		<.000
2 c2 &	MLocation ~ Genotype+Light +time+(1|Dish)+	**Genotype**		14.434	1		0.000
		Light		4.009	1		0.045
		Time		5.064	1		0.024
3 e & f	Percentage ~ state+light+Time +(1|date)	**State**		8.945	2		0.011
		Light		43.145	1		<.000
		Time		64.269	10		<.000
			♀ - ♂			1.153	0.481
			♀ - ☿			-	0.269
			♂ - ☿			-2.97	0.008
			Dark - light			6.568	<.000
3 g & h	Percentage ~ Sex+light+Time +(1|Date)	**Sex**		7.595	1		0.006
		Light		12.031	1		0.001
		Time		46.754	10		<.000
			♀ - ♂			-	0.006
			Dark - light			3.469	0.001
4 d	RematingLocation~Replacement+ Light+(1|Date)+(Time|Date)+(1|Dish)	**Replacement**		9.209	2		0.010
		Light		13.227	1		<0.00
			AA - Pep			1.36	0.362
			AA - AA &			-	0.134
			Pep - AA &			-	0.015
			Dark - light			3.637	0.000
5 c	MLocation~Light+time+(1|Dish)+ (1|Date)	Light		0.573	1		0.449
		Time		5.353	1		0.021