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Supplemental Information

Drosophila Aversive Behavior toward *Erwinia carotovora carotovora* Is Mediated by Bitter Neurons and Leukokinin

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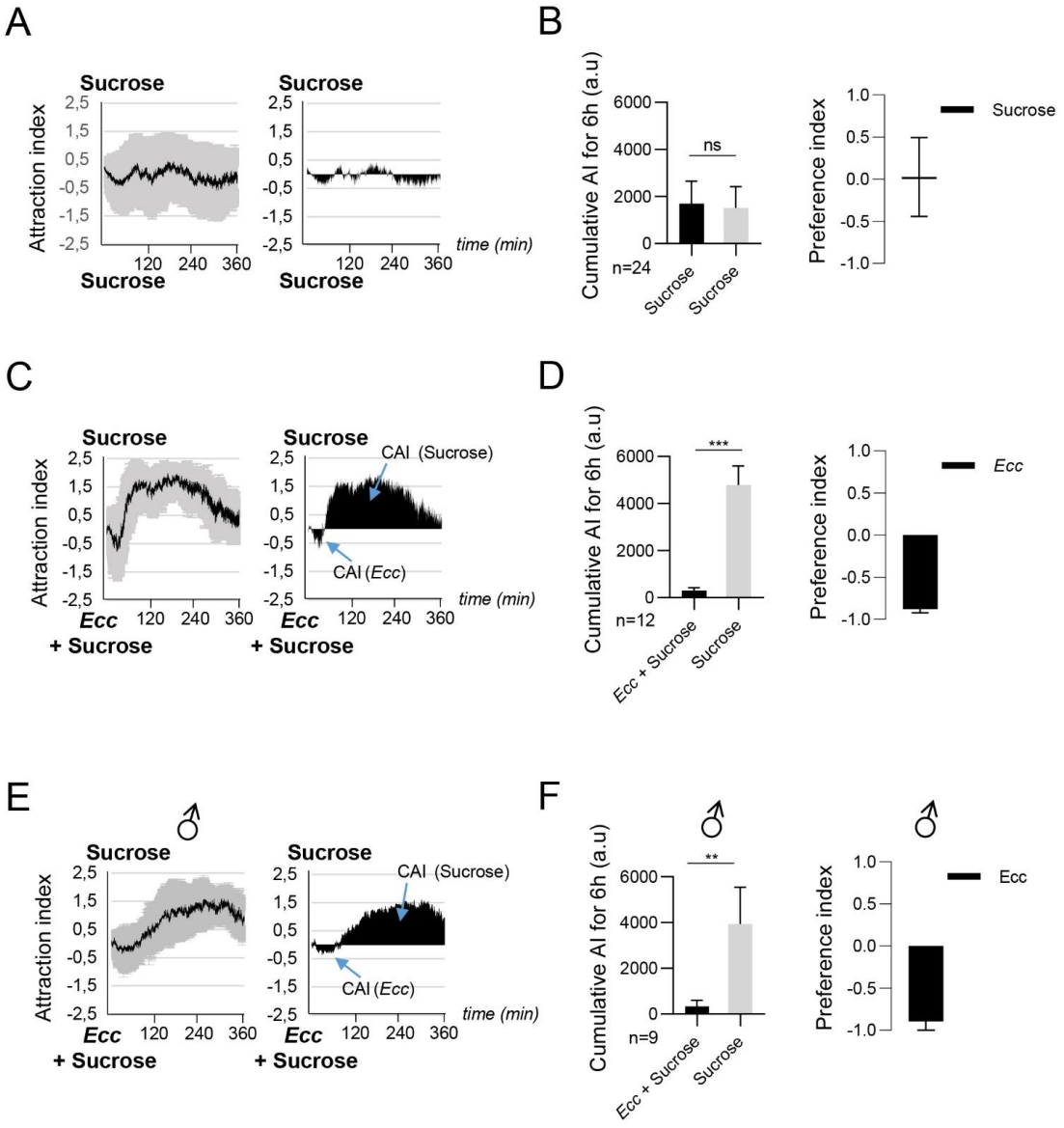

Figure S1. No directional bias in drop solution preference and *D. melanogaster* males behave as controls. Related to Figure 1.

A-B, No significant preference of adult females when given the choice between two identical 50mM sucrose solutions. A, (Left) Kinetic of the AI for sucrose in a sucrose versus sucrose experiment. (Right), CAI area for each sucrose solution and its distribution over time. B, (Left), histograms built with the CAI values from A. n.s: not significant. Wilcoxon matched-pairs signed-rank test, Two-tailed P value. Error bars correspond to standard deviation. The preference index for sucrose is calculated with the CAI values from B. C-D, Adult females displayed a two-step behavior when given the choice between an *Ecc* contaminated sucrose solution and a sucrose only solution of randomized position. C, (Left) Kinetic of the AI for sucrose in a sucrose versus sucrose + *Ecc* experiment. (Right), CAI area for each specified solution (arrows) and its distribution over time. D, (Left), histograms built with the CAI values from C.

*** P value< 0,001. Wilcoxon matched-pairs signed-rank test, Two-tailed P value. Error bars correspond to standard deviation. The preference index for *Ecc* is calculated with the CAI values from D. E-F, Adult males displayed a two-step behavior when given the choice between an *Ecc* contaminated sucrose solution versus sucrose only. E, (Left) Kinetic of the AI for Sucrose. Flies were first attracted by the bacteria solution before moving away from the contaminated solution. (Right) CAI area for each specified solution (arrows) and its distribution over time. F, Histograms built with the CAI values from E. ** P value< 0,01. Wilcoxon matched-pairs signed rank test, Two-tailed P value. Error bars correspond to standard deviation. The preference index for *Ecc* is calculated with the CAI values from F. For A, C and E left graphs, the black lines and the grey lines correspond respectively to the mean and the standard deviation, and for right graphs sole the mean value of the CAI obtained with multiple replicates is shown in black. n indicates the number of experimental replicates. a.u: arbitrary unit. Data are represented as mean +/- SD.

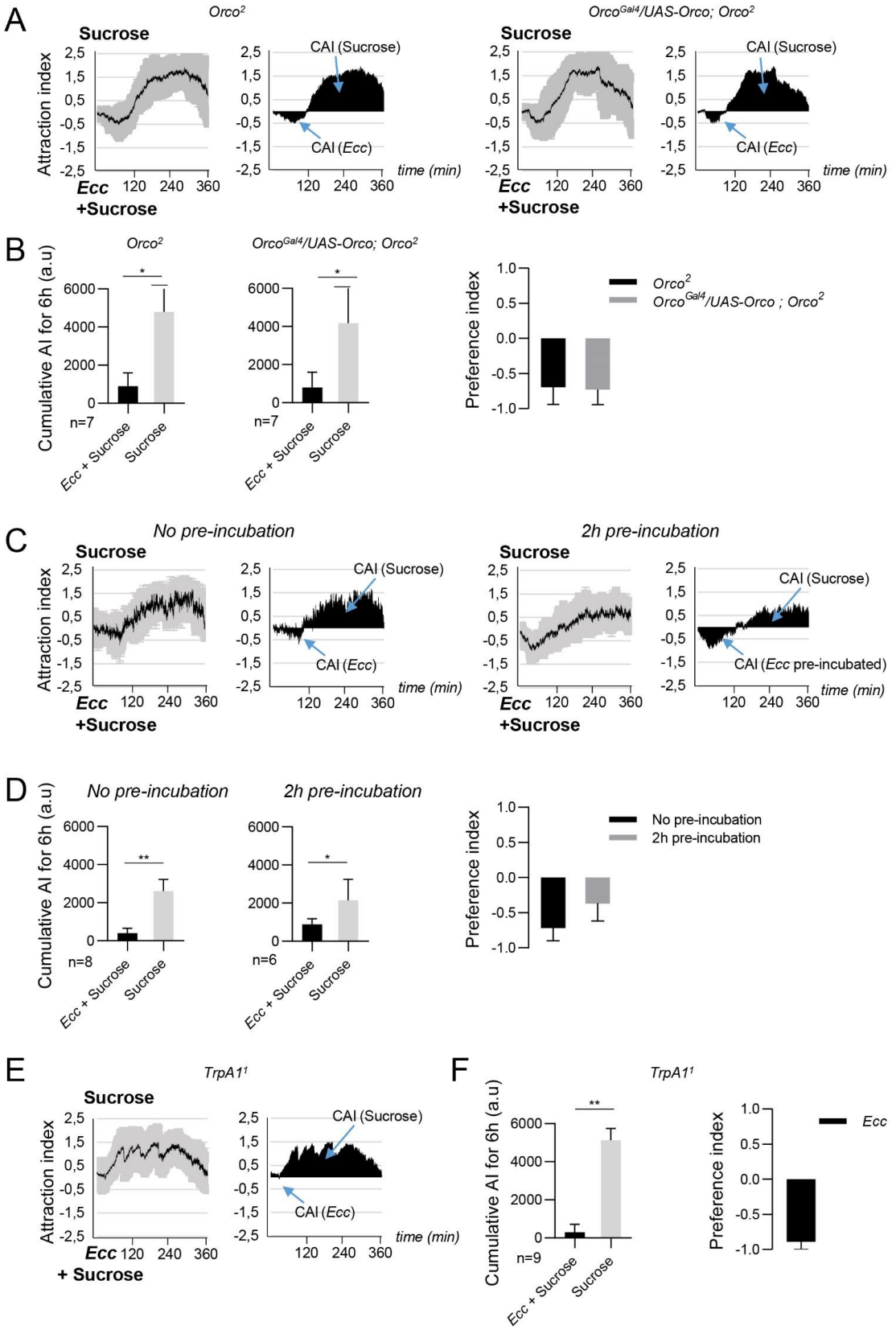

Figure S2. The odorant co-receptor Orco and the cation channel TrpA1 are not required for adult female's behavior to *Ecc*. Related to Figure 2.

A-B, *Orco*^{2} mutants and *Orco*^{2} rescued mutant (*Orco*^{Gal4}*/UAS-Orco; Orco*^{2}) displayed a two-step behavior. A, (Left graphs) Kinetic of the AI for sucrose in a sucrose versus sucrose + *Ecc* experiment. (Right graphs) CAI area for each specified solution (arrows) and its distribution over time. B, Histograms built with the CAI values from A. * P value< 0,05. Wilcoxon matched-pairs signed-rank test, Two-tailed P value. Error bars correspond to standard deviation. The preference indexes for *Ecc* are calculated with the CAI values from B. C-D, Pre-incubation of *Ecc* with sucrose does not affect the two-step behavior of adult females. C, (Left graphs) Kinetic of the AI for sucrose when flies were given the choice between an *Ecc* contaminated sucrose solution versus sucrose only or an *Ecc* contaminated sucrose solution pre-incubated 2h without flies versus sucrose only. (Right graphs) CAI area for each specified solution (arrows) and its distribution over time. D, Histograms built with the CAI values from C. * P value< 0,05 and ** P value< 0,01. Wilcoxon matched-pairs signed rank test, Two-tailed P value. Error bars correspond to standard deviation. The preference indexes for *Ecc* are calculated with the CAI values from D. E-F, Flies homozygotes for the loss of function allele *TrpA1*^{1} display aversion to *Ecc*. E, (Left graph) Kinetic of the AI index for Sucrose. (Right graph) CAI area for each specified solution (arrows) and its distribution over time. F, Histograms built with the CAI values from E. ** P value< 0,01. Wilcoxon matched-pairs signed rank test, Two-tailed P value. Error bars correspond to standard deviation. The preference index for *Ecc* calculated with the CAI values from F. For A, C and E left graphs, the black lines and the grey lines correspond respectively to the mean and the standard deviation, and for right graphs sole the mean value of the CAI obtained with multiple replicates is shown in black. n indicates the number of experimental replicates. a.u: arbitrary unit. Data are represented as mean +/- SD.

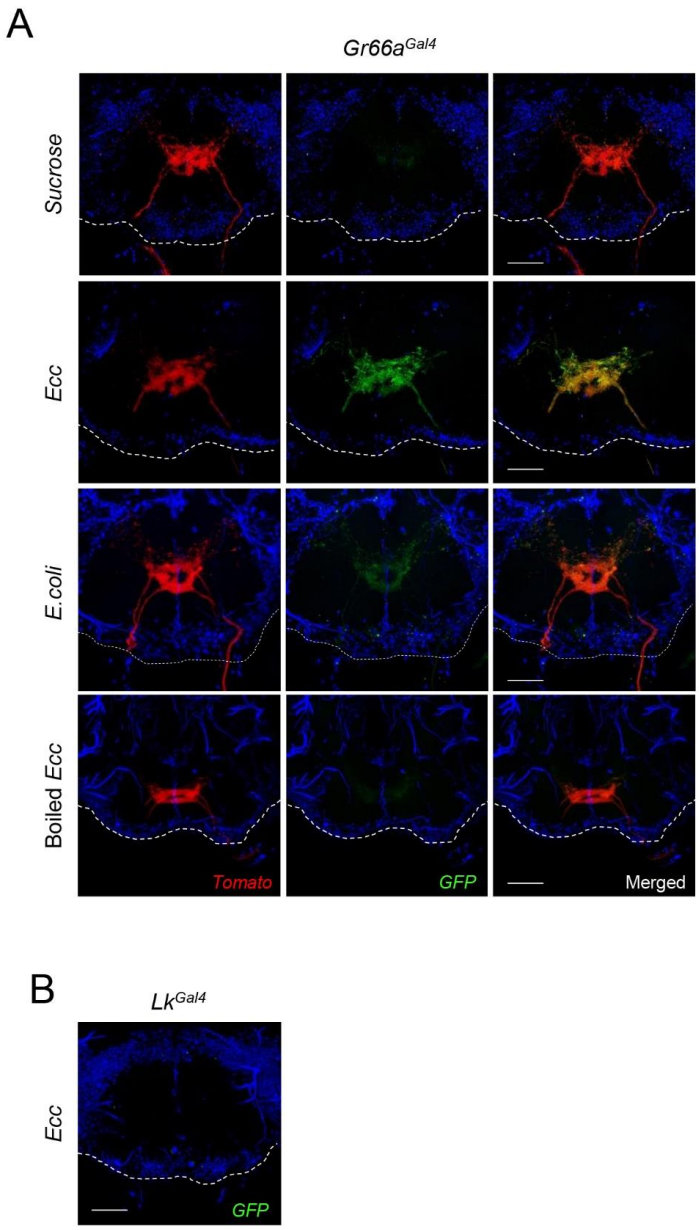

Figure S3. *Ecc* activates Ca^{+} release in *Gr66a* positive neurons but not in *Lk* positive ones. Related to Figure 5.

A, *Ecc*, and to a less extend *E. coli,* activates Ca^{+} release in Gr66a bitter neurons, but sucrose or boiled *Ecc* do not. Confocal images of the SEZ region of adult fly's brain of genotype *LexAop-CD8-GFP-2A-CD8-GFP; UAS-mLexA-VP16-NFAT/UAS-CD4::Tomato, lexAop-rCD2-GFP/Gr66a^{Gal4}*, that were fed with either Sucrose, *Ecc* + sucrose or *E.coli* + sucrose solutions. B, *Ecc* does not activates Ca^{+} release in Lk neurons. Confocal images of the SEZ region of adult fly's brain of genotype *LexAop-CD8-GFP-2A-CD8-GFP; UAS-mLexA-VP16-NFAT/UAS-CD4::Tomato, lexAop-rCD2-GFP/Lk^{Gal4}*, that were fed with either *Ecc* + sucrose solutions. For A and B, Red: Tomato, Green: GFP and Blue: nuclei staining with Hoechst. The dashed line is demarcating the brain periphery. Scale bar: 25 μm.

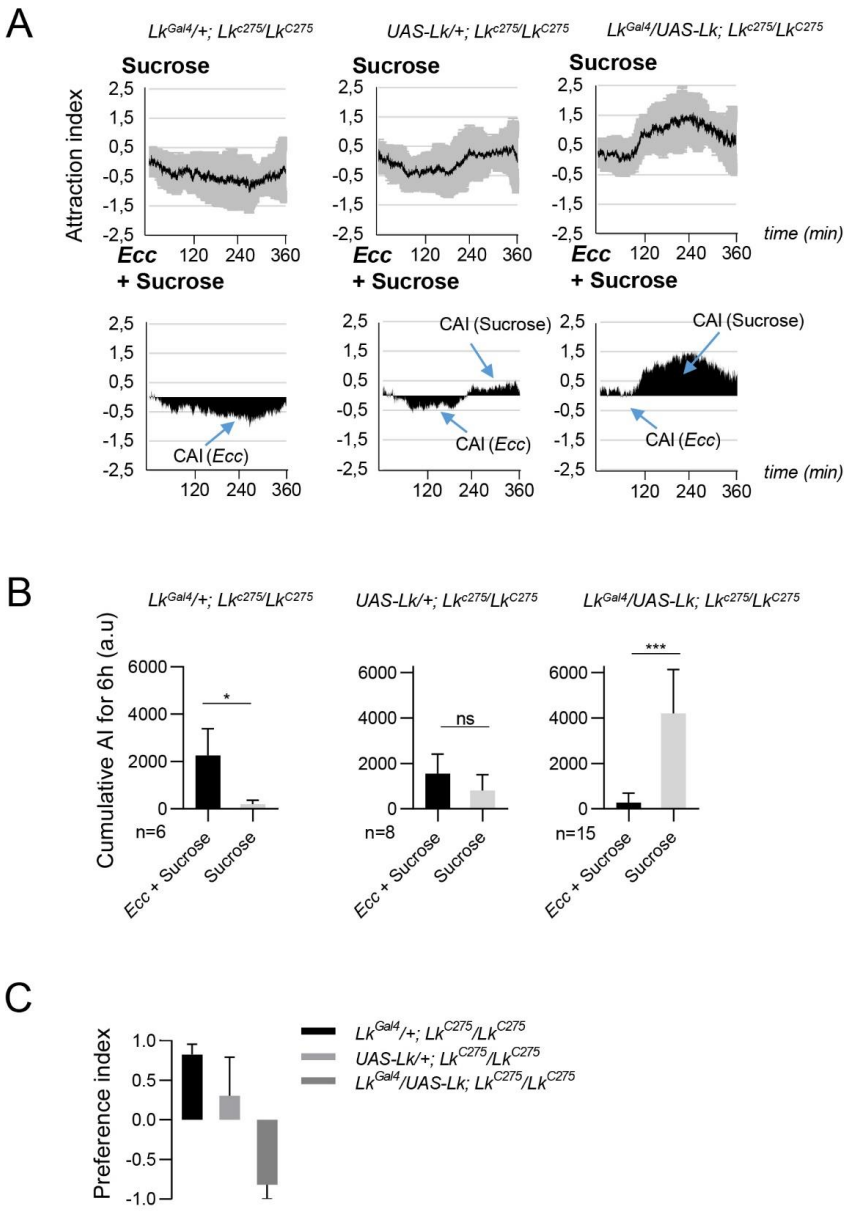

Figure S4. The neuropeptide Lk is required for the aversive perception of *Ecc.* Related to Figure 6.

A-B, Expression of *UAS-Lk* under the control of Lk^{Gal4} rescue the abnormal behavior observed for Lk^{c275} mutants. A, (Up) Kinetic of the AI for Sucrose. (Bottom) CAI area for each specified solution (arrows) and its distribution over time. B, Histograms built with the CAI values from A. * P value< 0,05 and *** P value< 0,001. ns, not significant. Wilcoxon matched-pairs signed rank test, Two-tailed P value. Error bars correspond to standard deviation. C, preference index for *Ecc* calculated with the CAI from B. A (top) the black lines and the grey lines correspond respectively to the mean and the standard deviation, and for bottom graphs, sole the mean value of the CAI obtained with multiple replicates is shown in black. n indicates the number of experimental replicates. a.u: arbitrary unit. Data are represented as mean +/- SD.

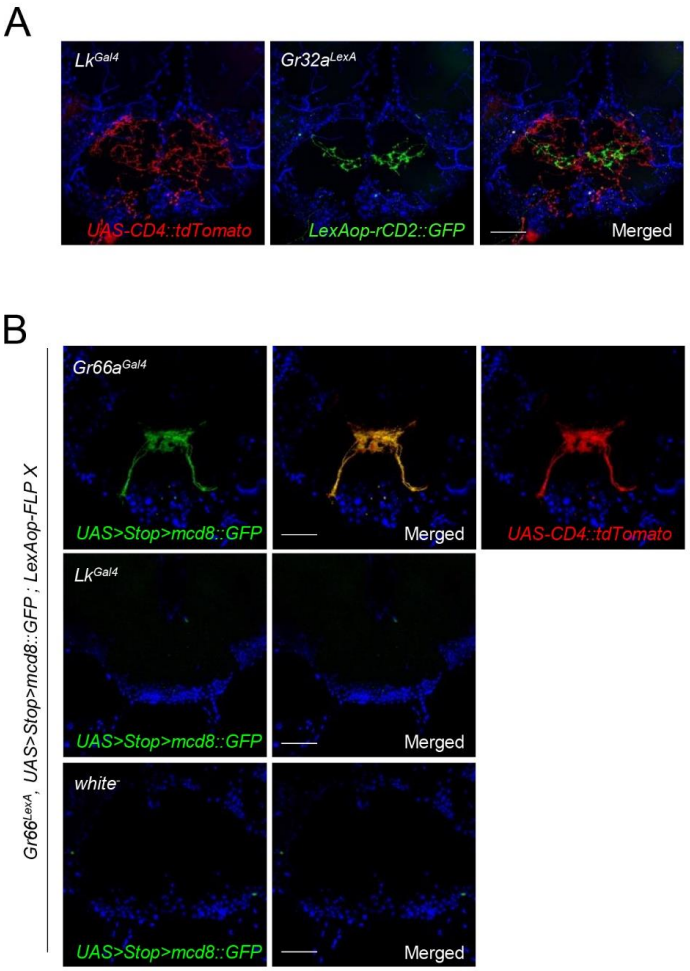

Figure S5. SEZ projections of Lk and bitter neurons are distinct. **Related to Figure 6.**

Confocal images of the SEZ region of adult brains showing that *Lk^{Gal4}* projections (red) do not co-localize with bitter neuron projections labelled with *Gr32^{LexA}* (green). Fly genotype is *Lk^{Gal4}/UAS-CD4::tdTomato ; Gr32^{LexA}/LexAop-rCD2::GFP*. B, leukokinin positive neurons do not share common identity with *Gr66^{LexA}* positive ones in the SEZ. Confocal images of the SEZ region of adult brains allowing activation of *UAS-mcd8::GFP* exclusively in Gr*66^{LexA}* positive cells (*Gr66^{LexA}, UAS>Stop>mcd8::GFP ; LexAop FLP*). As expected, crossing these flies to *Gr66^{Gal4}; UAS-CD4::tdTomato* flies, lead to *UAS-mcd8::GFP* expression in the SEZ (green) that perfectly match the expression of *UAS-CD4::tdTomato* (Red). No GFP positive cells are detectable using either *Lk^{Gal4}* or the control *white$^-$* fly strain. Blue: nuclei staining with Hoechst. Scale bar: 25 μm.

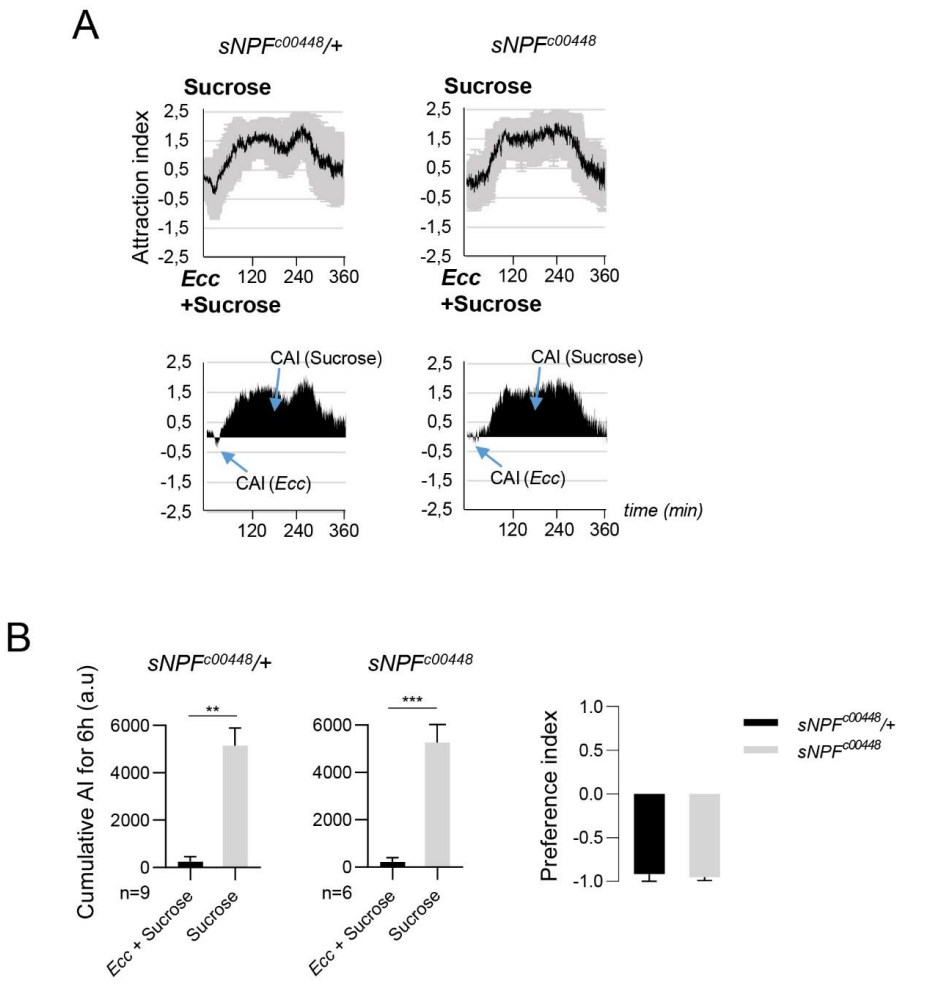

Figure S6. The short neuropeptide F is not required for the two-step behavior toward *Ecc.* Related to Figure 2.

A-B, *sNPF*^{c00488} mutants females displayed a normal two-step behavior as control *sNPF*^{c00488}/+ ones. A, (Top graphs) Kinetic of the AI for sucrose in a sucrose versus sucrose + *Ecc* experiment. (Bottom graphs) CAI area for each specified solution (arrows) and its distribution over time. B, Histograms built with the CAI values from A. ** P value< 0,01 and *** P value< 0,001. Wilcoxon matched-pairs signed-rank test, Two-tailed P value. Error bars correspond to standard deviation. The preference indexes for *Ecc* are calculated with the CAI values from B. For A top graphs, the black lines and the grey lines correspond respectively to the mean and the standard deviation, and for bottom graphs sole the mean value of the CAI obtained with multiple replicates is shown in black. n indicates the number of experimental replicates. a.u: arbitrary unit. Data are represented as mean +/- SD.

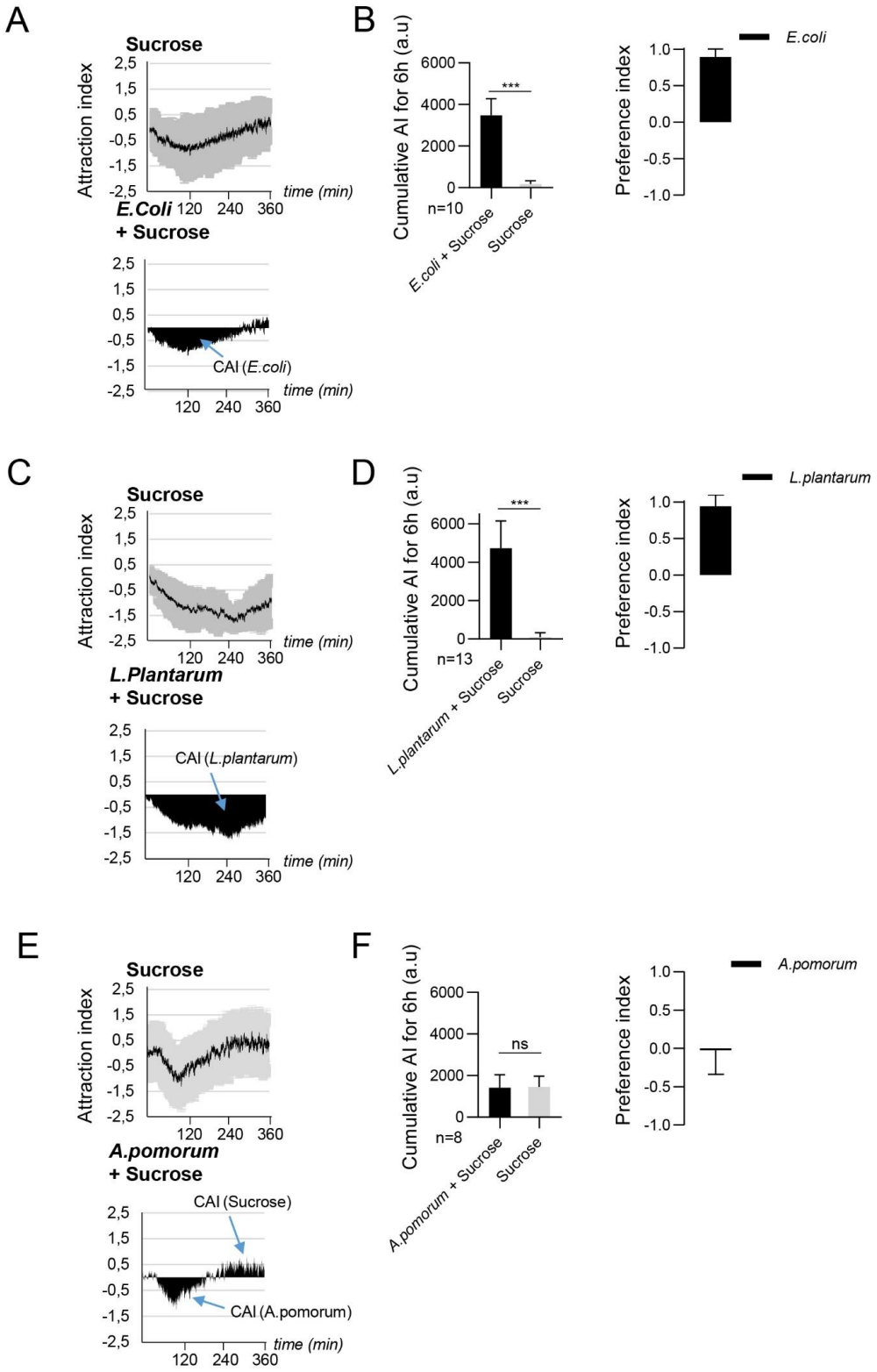

Figure S7. *D. melanogaster* adults display no aversion to either *E. coli*, *L. plantarum* or *A. pomorum* contaminated solutions. Related to Figure 2.

For A, C and E, the upper graphs show the kinetic of the AI for sucrose and the bottom graphs illustrate the CAI area for each solutions (arrows) and its distribution over time. For B, D and F, (left) graphs

correspond to histograms built with the CAI values from A, C and E, respectively. Right graphs are preference index for the bacteria contaminated solution calculated with the CAI values from B, D and F, respectively. A-B, Flies have a strong and statistically significant preference for sucrose + *E. coli* versus Sucrose. *** P value< 0,001. Wilcoxon matched-pairs signed rank test, Two-tailed P value. Error bars correspond to standard deviation. C-D, Flies had a strong and statistically significant preference for sucrose + *L. plantarum* versus Sucrose. *** P value< 0,001. Wilcoxon matched-pairs signed rank test, Two-tailed P value. Error bars correspond to standard deviation. E-F, flies had no preference for sucrose + *A.pomorum* versus Sucrose. ns, not significant. Wilcoxon matched-pairs signed rank test, Two-tailed P value. Error bars correspond to standard deviation. For A, C and E (top): the black lines and the grey lines correspond respectively to the mean and the standard deviation, and for bottom graphs, sole the mean value of the CAI obtained with multiple replicates is shown in black. n indicates the number of experimental replicates. a.u: arbitrary unit. Data are represented as mean +/- SD.

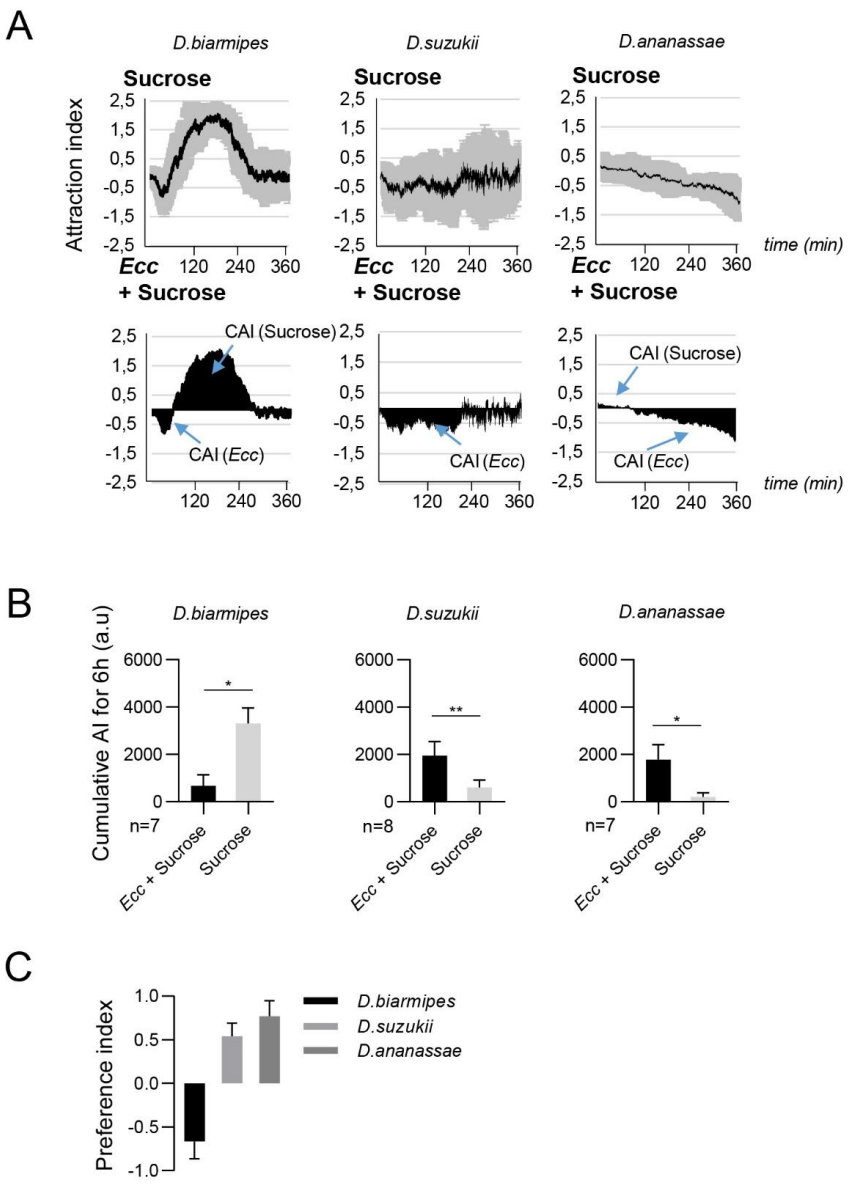

Figure S8. *D. melanogaster* behavior towards *Ecc* is species-specific. Related to Figure 2.

A, (Up) Kinetic of the AI index for Sucrose. (Down) CAI area for each specified solution (arrows) and its distribution over time. B, Histograms built with the CAI values from A. *D. biarmipes* have a strong and statistically significant preference for sucrose versus sucrose + *Ecc* while *D. suzukii* and *D. ananassae* prefer the contaminated solution versus Sucrose. * P value< 0,05 and ** P value< 0,01. Wilcoxon matched-pairs signed rank test, Two-tailed P value. Error bars correspond to standard deviation. C, preference index for *Ecc* calculated with the CAI values from B. A (top) the black lines and the grey lines correspond respectively to the mean and the standard deviation, and for bottom graphs, sole the mean value of the CAI obtained with multiple replicates is shown in black. n indicates the number of experimental replicates. a.u: arbitrary unit. Data are represented as mean +/- SD.

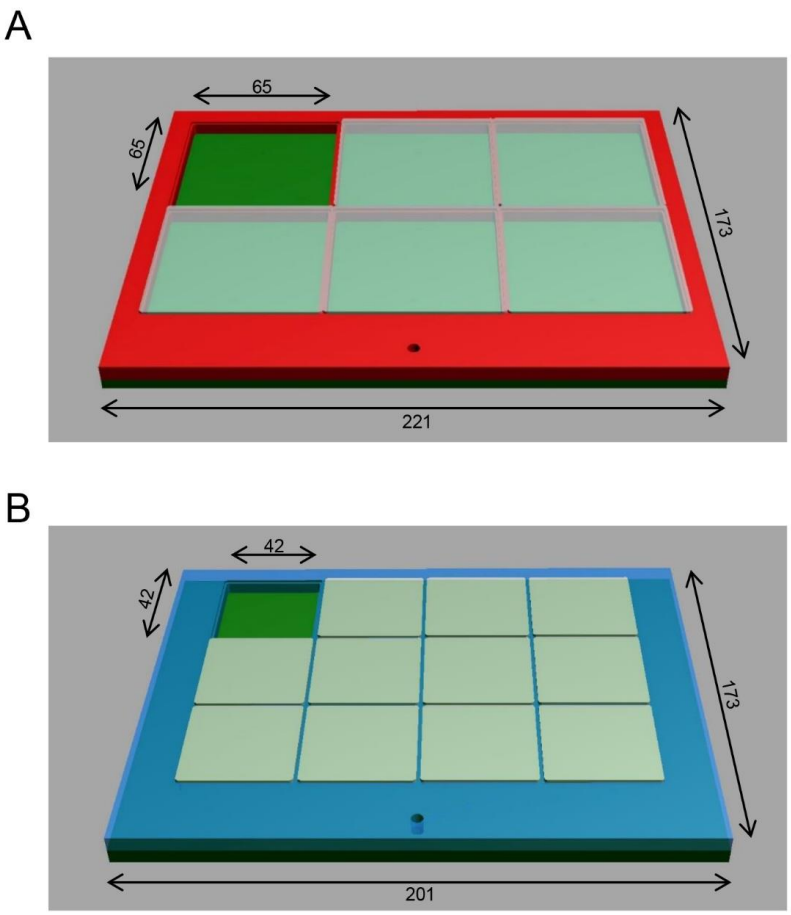

Figure S9. Dimensions of the apparatus used for behavioral assays. Related to STAR Methods.

A-B, Cartoon of the 6 arenas and the 12 arenas apparatus with the dimensions indicated in millimeters. Each apparatus is composed of three distinct plastic parts, the bottom part (dark green) which is a flat plain slab on top of which is glued the plastic grid containing 6 (red in A) or 12 (blue in B) squared holes and the 6 (or 12) removable plastic caps (light green in A and B) used to cover the arenas. The holes shown in A and B is used to screw the plastic arm (not shown here) design to fix the camera on top of the apparatus.

Transparent Methods

D.melanogaster strains and maintenance

The strains used in this work are: w^{1118} BL#3605, *CantonS* BL#64349, $orco^{2}$ BL#23130, $Orco^{Gal4}$ (Larsson et al., 2004), *UAS-Orco* BL#23145, $Relish^{E20}$ (Hedengren et al., 1999), *Df(3R)ED5301* BL#9225, $Gr66a^{Gal4}$ BL#57670, *UAS-Kir2.1::EGFP* BL#6595, $Gr66a^{ex83}$ BL#25027, $Gr66a^{+t8}$; $Gr66a^{ex83}$ BL#35528, $Gr33a^{1}$ BL#31425, *Tl*{*Gal4*}$Gr33a^{Gal4}$ BL#31427, $Gr33a^{1}$; UAS-*Gr33a* BL#31424, Lk^{C275} BL#16324, $G\alpha q^{1}$ BL#42257, $TrpA1^{1}$ BL#26504, *UAS-CD4::tdTomato* BL#35841, *UAS-CaLexA* BL#66542, Lk^{Gal4} BL#51993, *UAS>Stop>mcd8::GFP* BL#30125, $Gr66a^{LexA}$ (from Kristin Scott), $Gr32a^{LexA}$ (from Anupama Arun Dahanukar), *LexAop-FLP* BL#55819, *LexAop-rCD2::GFP* BL#66544, *UAS-Lk* (this work, molecular detail of the construct under request) $Diptericin\text{-}Cherry^{C1}$ (Charroux and Royet, 2009), $PGRP\text{-}LB^{\Delta}$ (Paredes et al., 2011), $Gr5a^{Gal4}$ BL#57591, $R1Gr5a^{LexA}$;; $\Delta Gr61a,\Delta Gr64a\text{-}f$ and $Gr43a^{-}$ (*Tl{GAL4}*$Gr43a^{GAL4}$) (from Hubert Amrein), $Gr63a^{1}$ BL#9941, $UAS\text{-}Oct\text{-}TyrR^{RNAi}$ BL# 28332 and $sNPF^{c00448}$ (from Michael D. Gordon). Flies were grown at 25°C on a yeast/cornmeal medium in 12h/12h light/dark cycle-controlled incubators. For 1liter of food, 8.2g of agar (VWR, cat. #20768.361), 80g of cornmeal flour (Westhove, Farigel maize H1) and 80g of yeast extract (VWR, cat. #24979.413) were cooked for 10 min in boiling water. 5.2 g of Methylparaben sodium salt (MERCK, cat. #106756) and 4 ml of 99% propionic acid (CARLOERBA, cat. #409553) were added when the food had cooled down. For antibiotic (ATB) treatment, the standard medium was supplemented with Ampicillin, Kanamycin, Tetracyclin and, Erythromycin at 50 µg/ml final concentrations.

Imaging

Adult tissues were dissected in PBS, fixed for 20 min in 4% paraformaldehyde on ice and rinsed 3 times in PBT (PBS + 0.1% Triton X-100). The tissues were mounted in Vectashield (Vector Laboratories) fluorescent mounting medium, with or without DAPI. Images were captured with an LSM 780 Zeiss confocal microscope.

Bacterial strains

The following microorganisms were used: *Erwinia carotovora carotovora 15* 2141 (grown at 30°C), *Erwinia carotovora carotovora 15 pOM1-GFP* (grown at 30°C), $Lactobacillus\ plantarum^{WJL}$ (grown at 37°C), *Escherichia coli* strain DH5 α (grown at 37°C) and *Acetobacter pomorum* (grown at 30°C). Bacteria were cultured overnight in Luria-Bertani (for *Ecc*, *Ecc-GFP* and *E. coli*) or MRS medium (for *L.plantarum* and *A. pomorum*). Bacterial cultures were centrifuged at 4000 g for 15 min at RT and re-suspended in 1XPBS. Cells were serially diluted in PBS and their concentration was determined by optical density (OD) measurement at 600 nm.

Fly preparation and chemical used in behavioral assays

We used 4-6 days old flies raised at 25°C in presence of ATB in the food. Flies were starved during 16h before the behavioral assay using empty vial with no food closed by a plug soaked with 500 µl of water. 10 to 20 flies were anesthetized on the ice for 5 minutes and loaded in each arena of our apparatus, where two drops of a given solution were previously deposited. We used the following compound from Sigma-Aldrich: sucrose (cat #S1888), caffeine (cat #C0750) and Eriauglaucine blue (Sigma-Aldrich, cat #861146) at 125 µg/ml final concentration to color the liquid solutions. All behavioral experiments using bacteria were performed with bacteria diluted at OD_{600}=50 in 50 mM sucrose (excepted for Figure 7E where a serial dilution of *Ecc* in 50 mM sucrose was used). For *Ecc* heat inactivation, a solution of *Ecc* diluted in 50 mM sucrose (final OD_{600}=50) was incubated at 96°C for 20 minutes, then cool down before use. All experiments were performed in a behavioral room with constant temperature (24°C) and humidity (65%). The dimensions of the apparatus used for behavioral assays (the 6 arenas and the 12 arenas apparatus) are shown in Figures S9A-B.

Bacterial Load Analysis

Bacterial load of surface-sterilized individuals was quantified by plating serial dilutions of lysates obtained from a single individual on a nutrient agar plate. Biological triplicates were collected for each experimental condition. Homogenization of individuals or tissues was performed using the Precellys 24 tissue homogenizer (Bertin Technologies, France) and 0.75/1 mm glass beads in 800 ml of the appropriate bacterial culture medium.

Adult oral infection

We used 4-6 days old female raised at 25°C in presence of ATB in the food. 24h before the infection, female flies were transferred in vials without ATB and then placed in a fly vial with *Ecc* contaminated food. The food solution was obtained by mixing a pellet of an overnight culture of bacteria *Ecc-15* (OD=200) with a solution of 5% sucrose (50/50) and added to a filter disk that completely covered the agar surface of the fly vial. Flies were incubated at 25°C for 24h.

Statistical Analysis

The Prism software (GraphPad) was used for statistical analyses. We used the nonparametric Wilcoxon matched-pairs signed-rank test. P value was indicated as follow: * for P<0,05, ** for P<0,01, *** for P<0,001. ns for not significantly different.

Flybox

For video/frame processing and analysis, we have developed a homemade software called *Flybox* which can track up to six different experiment boxes simultaneously. Video/frame processing and analysis have been achieved using MATLAB R2015B, Statistical Toolbox and, Image Analysis Toolbox.

Given a movie M:

$$M = (F_0, ..., F_n)$$

where n denotes the number of frames into M, every frames of M are composed of three-color components (RGB)

$$F_k = (R_k, G_k, B_k) \in M$$

where k stands for the kth frame into the movie M

Droplet solution detection and localization

As the size and shape of the two droplet solutions per box were possibly evolving throughout the whole movie depending on fly appetite, we decided to take only the first video frame as a reference for the subsequent droplet solution detection and localization analysis and a user input feedback to assign a qualitative label to every droplet solution content. As a preprocessing step, we converted the original frame color space from RGB to HSV (Hue Saturation Value) to keep only the saturation as the color intensity of the two droplets was different from the background into this component. We used a bit depth value *b* of 255 (8-bits) to rescale pixel values to be in the 0-1 range.

$$F_0' = \frac{F_0}{b}$$

We calculated the saturation component of the reference frame as:

$$S = \begin{cases} 0 & \text{if} \quad max(F_0') = 0 \\ 1 - \frac{min(F_0')}{max(F_0')} & \text{otherwise} \end{cases}$$

We binarized this image to create the droplet masks by using a binarization threshold value α to 0,5.

$$B_{droplets} = \begin{cases} 0 & \text{if} \quad S < \alpha \\ 1 & \text{otherwise} \end{cases}$$

To extract droplets from this binary mask, we extracted all 8-connected components, and we discarded those having less than 50 pixels as total area to avoid false positive detections and keeping only the two true positive droplets into two individual masks D1 and D2:

$$(D_1, D_2) = ConnComp(B_{droplets})$$

We then extracted droplet centroids:

$$c_{D_k} = \left(\frac{1}{n} \sum_{i=1}^{n} x_i \quad ; \frac{1}{n} \sum_{i=1}^{n} y_i \right) ; (x_i, y_i) \in D_k$$

and we assigned them their corresponding user-defined label.

Fly detection and localization

The fly detection process is achieved for each video frame independently and into every experimental box simultaneously. Starting from the original RGB image, we subtracted the red from the green component to be able to decrease the blueish color of the droplet solution without altering the fly original blackish colors.

$$H_k = G_k - R_k$$

We then applied a Gaussian smoothing filter using σ =2 to both hide small image artifacts and unsharp fly bodies to help in their further detection.

$$Gauss_k = \frac{1}{2\pi\sigma^2} e^{\frac{-(x^2+y^2)}{2\sigma^2}}; (x,y) \in H_k$$

We noticed that pixels coming from the background are both overrepresented and have a rather uniform pixel intensity which is not the case of pixels coming from fly bodies which are underrepresented. To easily discriminates the two sets of pixels, we decided to rescale every pixel intensity using a z-score scaling procedure by keeping only ones having a large standard deviation (z-score>11).

$$B_{flies} = \begin{cases} 0 & \text{if} \quad \frac{Gauss_k - \mu(Gauss_k)}{\sigma(Gauss_k)} < 11 \\ 1 & \text{otherwise} \end{cases}$$

We extracted all 8-connected components as individual flies:

$$(Fly_1, ..., Fly_m) = ConnComp(B_{flies})$$

and we calculated centroid coordinates for every detected fly:

$$c_{Fly_k} = \left(\frac{1}{n} \sum_{i=1}^{n} x_i \quad ; \frac{1}{n} \sum_{i=1}^{n} y_i \right); (x_i, y_i) \in Fly_k$$

Quantifying the droplet solution preference for the overall fly populationWe started from the hypothesis that a population of fly attracted by one particular droplet solution should spend more time close to it relative to the other one. First, we calculated an *attraction index* as the log2 ratio of the sum of Euclidean distance of flies to each droplet:

$$attractivity(F_k) = log_2 \left(\frac{\sum_{k=1}^{m} \| c_{D1} - c_{Fly_k} \|}{\sum_{k=1}^{m} \| c_{D_2} - c_{Fly_k} \|} \right)$$

As a result, for a given frame, we can measure the overall attraction of one particular droplet solution relative to the other: D2 attraction will be translated into a positive index value and D1 by a negative. The strength of attraction can also be assessed as the absolute difference between the index value and zero value: high index values (positive or negative) are then correlated with high attraction (resp. low index for low attraction) Then, for every droplet, D1 and D2, we independently calculated a *Cumulative Attraction Index* (CAI) as the total area under the curve of either each negative or each positive

attraction *indexes* throughout the whole movie. To differentiate the two cases (negative and positive attraction indexes), we first defined two logical functions *pos* and *neg* as:

$$pos(F_k) = \begin{cases} 1 & \text{if} \quad attractivity(F_k) > 0 \\ 0 & \text{otherwise} \end{cases}$$

$$neg(F_k) = \begin{cases} 1 & \text{if} \quad attractivity(F_k) < 0 \\ 0 & \text{otherwise} \end{cases}$$

and finally, *the preference index* (named CAI in the main text) as the trapezoidal numerical integration of attraction indexes calculated throughout the whole movie:

$$preferenceD_2(M) \approx \frac{1}{2n} \sum_{i=1}^{n} (pos(F_i - 1) \cdot attractivity(F_i - 1) + pos(F_i) \cdot attractivity(F_i))$$

Supplemental References

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