Oviposition preference for and positional avoidance of acetic acid provide a model for competing behavioral drives in *Drosophila*

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Selection of appropriate oviposition sites is essential for progeny survival and fitness in generalist insect species, such as *Drosophila melanogaster*, yet little is known about the mechanisms regulating how environmental conditions and innate adult preferences are evaluated and balanced to yield the final substrate choice for egg-deposition. Female *D. melanogaster* are attracted to food containing acetic acid (AA) as an oviposition substrate. However, our observations reveal that this egg-laying preference is a complex process, as it directly opposes an otherwise strong, default behavior of positional avoidance for the same food. We show that 2 distinct sensory modalities detect AA. Attraction to AA-containing food for the purpose of egg-laying relies on the gustatory system, while positional repulsion depends primarily on the olfactory system. Similarly, distinct central brain regions are involved in AA attraction and repulsion. Given this unique situation, in which a single environmental stimulus yields 2 opposing behavioral outputs, we propose that the interaction of egg-laying attraction and positional aversion for AA provides a powerful model for studying how organisms balance competing behavioral drives and integrate signals involved in choice-like processes.

Oviposition provides a powerful yet simple means for monitoring preference behavior in *Drosophila melanogaster*, since a laid egg represents a marker for female position. Past studies have used egg laying as a readout for conditions advantageous to progeny development (1, 2), in which oviposition preference effectively separates larvae of different sibling species of *Drosophila*. Egg laying has also been used to detect aversion toward compounds toxic to both larvae and adults (3, 4). Furthermore, numerous studies have used patterns of oviposition to distinguish subtle differences in host plant preferences, which have provided insights into resource requirements and ecological behaviors of different *Drosophila* species (5, 6).

Despite numerous studies using oviposition-site selection as a behavioral readout, direct study of the relevant sensory circuits and the oviposition program itself have been initiated only recently in *D. melanogaster* (7, 8). To investigate the genetic mechanisms and neural circuits regulating this important behavioral choice in *D. melanogaster*, we developed a simple yet robust 2-choice assay that utilizes acetic acid (AA), a naturally occurring product of fruit fermentation, as an egg-laying attractant (9, 10). However, in addition to verifying a strong egg-laying preference for AA, we surprisingly observed *D. melanogaster* show a strong positional aversion to the same AA-containing food. We demonstrate that when sampling for oviposition sites, females integrate input from distinct sensory modalities to choose a particular behavioral output from 2 competing options: ovipositional attraction for and positional repulsion to AA. Egg-laying preference is primarily relayed through gustatory neurons, while positional aversion is relayed through the olfactory system. We also map central brain regions mediating these competing behaviors. Taken together, the process by which females integrate sensory information to execute these competing and interacting behaviors provides a tractable model for studying choice-like behavior in *D. melanogaster*.

**Results**

**Egg-Laying Preference for and Positional Aversion to AA-Containing Food.** To investigate the mechanisms involved in egg-laying preference, we devised a simple apparatus in which females are allowed the choice to lay eggs on regular food or food containing various concentrations of AA (Fig. 1A). Similar to previous observations (9, 10), mated females laid approximately 91% of their eggs on food containing 5% AA (Fig. 1B and D; + AA) as compared to regular food (Fig. 1B and D; −AA), with an oviposition index (OI) of +0.82. It has been postulated that *D. melanogaster* may use AA as an energy source (11), such that oviposition preference would result from an attraction to AA-containing media as a feeding source. To test this hypothesis, we first observed the physical location of flies during the 3-h oviposition assay. Surprisingly, females avoided food containing 5% AA (the concentration found naturally in vinegar), with a position index (PI) of −0.33 (Fig. 1B and E). To test for feeding preferences, we used a modified 2-choice assay in which different food dyes were mixed into the halves of the dish. After a sampling period, gut contents were analyzed by thin-layer chromatography (TLC) to quantify the relative ingestion of each dye. Flies ingested essentially equal amounts of food containing or lacking AA (Fig. 1C). Thus, oviposition-site selection does not reflect innate positional or feeding preferences, and may be in direct conflict with positional preference under ecologically relevant conditions. Recent studies show similar decoupling between adult taste and egg-laying preferences (7, 12).

Interestingly, positional repulsion for AA-containing food was stronger in virgin females and males (Fig. 1B). Since virgin females lay fewer eggs than mated females (Fig. S1.4), they likely search for egg-laying substrates less frequently, and may therefore have less incentive to overcome their innate positional aversion to AA-containing food. Males explore AA-containing food even less frequently than virgin females. Thus, the positional aversion to AA grows as the need to lay eggs is diminished or absent, implying that the attractive oviposition and repulsive positional drives are in competition. However, mated and virgin females showed equivalently high OI values in response to AA-containing food (Fig. 1B); attraction to AA as an oviposition

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substrate is therefore an innate preference not affected by post-mating behavioral modifications (13).

To determine if oviposition preference and positional aversion were specific to AA or elicited by the acidity of AA-containing food, we analyzed these behaviors on foods containing acetic, hydrochloric (HCl), or sulfuric (H2SO4) acids, titrated to equivalent pH values. At pH 3.5 (5% AA), females showed negligible oviposition preference for foods with HCl or H2SO4, while preference for AA-containing food was high (Fig. 2B). Likewise, the positional aversion observed with 5% AA was eliminated at pH 3.5 (5% AA) and at the equal pH values. At pH 3.5 with AA, HCl, or H2SO4. Control H2O-supplemented food has pH ~ 4.5. Significant responses at pH ~ 3.5 were only observed for AA-containing substrate (***, P < 0.01; 1-way ANOVA, Bonferroni post-test; n = 9). There were significant differences between the dose-response curves for AA when compared with HCl and H2SO4 (linear regression; ***, P < 0.0001; n = 4–8).

Fig. 1. Egg-laying and positional responses to acetic acid (AA). (A) Apparatus for assaying oviposition and positional preference. Mated females were presented a dish in which one-half contains food mixed with a compound of choice, and the other contains food mixed with an equivalent volume of water. An oviposition preference index (OI) and a positional preference index (PI) were calculated during the 3-h sampling period (see Methods for OI and PI formulas). (B) Comparison of OI and PI values of mated females, virgins, and males in response to 5% AA. As the egg-laying rate decreased for each consecutive group (left-to-right, shaded triangle), the positional aversion response increased (*, P < 0.05; ***, P < 0.01; 1-way ANOVA, Bonferroni post-test; n = 17). No significant differences in OI values were observed between female groups (Student’s unpaired t test). (C) Females showed no preference for consuming food containing AA. Mated females were presented the following 2-choice food combinations: −AA/−AA, Blue #1/Green #3 (black bar); +AA/−AA, Blue #1 + 5% AA/Green #3 (green bar); −AA/+AA, 5% AA/Blue #1/Green #3 (blue bar). After feeding, the amount of dye in fly gut contents was quantified by TLC. (D) Females deposited the majority of eggs on 5% AA substrate (+AA). (E) A single time point showing females spending time on media lacking AA. Photos were taken after 3 h (D) and at the 1-h time point (E). To facilitate photography in D and E, 0.5% agarose was used instead of regular food.

The Olfactory System Mediates Positional Aversion to AA. Although the sensory inputs and genetic pathways involved in D. melanogaster oviposition preference are relatively uncharacterized, the role of taste and olfaction in egg laying of other insects has been investigated (1, 6, 14, 15). In addition, AA can be aversive to D. melanogaster in certain olfactory assays (5, 16). We therefore analyzed the behavior of flies with impaired or enhanced olfaction. To impair olfaction, we surgically removed the primary olfactory organs, the third antennal segments (17, 18). Antennaectomized females, while normal for egg-laying preference (Fig. 3A), lost their positional aversion to 5% AA (Fig. 3B). Thus, olfaction is essential for positional aversion to AA, but is not required for oviposition preference. Consistent with these data are our observation that silencing antennal projection neurons disrupted positional aversion (Table S1). Males lacking antennae also showed diminished aversion to 5% AA (Fig. S3C).

To analyze the effect of enhanced olfactory input, we tested 1) mutant flies with an increased sense of smell and 2) wild-type flies exposed to higher AA concentrations. Mutations in white rabbit (whir) show an elevated olfactory startle response to ethanol and other odorants (19) and are suspected to possess an
enhanced sense of smell. Consistent with this hypothesis, position
aversion to AA was increased in whir1 females, an effect that
was significantly diminished by antennal removal (Fig. 3B).
Furthermore, the increased positional repulsion exhibited by
whir1 females was accompanied by egg-laying aversion for AA-
containing food; this effect was also strongly ameliorated by
antennaectomy (Fig. 3A). Similarly, removing the antenna of
whir1 males reduced their excessive positional repulsion to AA
(Fig. S5C). We next tested responses to a high concentra-
tion (10% AA), which normally eliminates oviposition preference
and enhances positional aversion (Fig. S5 A and B). Removing
antennae restored egg-laying preference to nearly normal levels
and normalized positional aversion (Fig. S5 A and B). Positional
aversion was not completely eliminated in antennaeectomized
whir1 females and wild-type flies exposed to 10% AA (Fig. S5 B
and C), suggesting that either olfactory neurons on the maxillary
palps or other sensory modalities are engaged at high AA
concentrations. Despite this caveat, our data show that olfactory
neurons in the third antennal segment are the primary sensors
inducing positional aversion to AA.

To further show that oviposition and positional preferences
are competing drives, we asked if reduced olfactory input would
increase egg-laying preference for AA. Because OIs approach
saturation at 5% AA (Fig. 3A), an increase would be concealed
by a “ceiling effect.” We therefore analyzed responses to 0.25%
AA, a concentration that yielded a moderately attractive egg-
laying response and no positional avoidance (OI = +0.34, PI = 
−0.03; Fig. S5D). Antennaectomized females exhibited
increased egg-laying preference and a small but significant shift to
a more positive positional preference (OI = +0.55, PI = +0.10; 
Fig. S5D). Thus, even low AA concentrations are detected by
the olfactory system and perceived as slightly repulsive. These data
support our hypothesis that olfactory-based aversion competes
with egg-laying attraction for AA.

The Gustatory System Mediates Oviposition Attraction to AA. Our
data indicated that a sensory modality other than olfaction
mediates egg-laying preference; a likely candidate was the
gustatory system. Gustatory bristles are present on the primary
taste structures: the labellum, front legs, wing margins, and the
ovipositor (20). To test if gustatory neurons mediate egg-laying
preference, we assayed pox-neuro (poxn) mutants, in which taste
bristles are transformed into mechanosensory bristles lacking
gustatory receptors (21, 22); null mutants also have defects in the
central nervous system (23). Homozygous poxnnull flies showed
reduced egg-laying preference (OI = +0.28, PI = +0.51; Fig. 4)
when compared to wild-type, poxnheterozygous, and
poxnmutant homozygous flies carrying the SuperA transgenic
construct (23) that rescues all poxn defects. These data implicate
taste receptors in the egg-laying attraction for AA. However,
positional aversion was also reduced in heterozygous poxnmutant
females (PI = 0.09; Fig. 4B), likely due to abnormalities in
olfactory processing centers in the mutant (23). To overcome
these issues, we tested transgenic strains in which poxn expres-
sion was restored in a tissue-specific manner. The full-1 and f152
transgenes restore normal brain morphology and chemosensory
bristles to poxnmutant flies, except for taste organs found on the
labellum (23). poxnnull females carrying the full-1 or full-152
transgene showed diminished AA egg-laying preference (OI = 
+0.12, +0.23, respectively; Fig. 4A), but still maintained a robust
positional aversion to 5% AA (PI = −0.47, −0.42, respectively; 
Fig. 4B). In fact, positional aversion to 5% AA was enhanced
when compared with control strains. To confirm that gustatory
and not olfactory pathways mediate egg-laying responses to AA,
we removed the third antennal segments from the poxn-rescue
lines. As expected, antennaectomized flies showed reduced
positional aversion to AA, while oviposition indices were un-
changed (Fig. 4). Overall, these data show that females use taste
neurons on the labellum to recognize AA as an egg-laying
attractant, and that reduced egg-laying preference leads to a
compensatory increase in positional repulsion.

Brain Centers Involved in Egg-Laying and Positional Preferences for
AA. Thus far, our data has identified peripheral sensory systems
that induce egg-laying and positional responses to AA, and
shown that behavioral outputs of the 2 preference pathways are in competition. To identify higher-order brain regions that may mediate and integrate signals from these competing pathways, we silenced specific neuronal populations by expressing a temperature-sensitive Shibire transgene, UAS-ShiP2-120 under the control of various GAL4 lines. 58 GAL4-expressing lines were crossed to UAS-ShiP2, and their progeny were assayed for egg laying and positional preferences at the permissive (23 °C) and restrictive (30 °C) temperatures (Table S1).

Three GAL4 lines with highly selective expression in the mushroom body (MB) lost egg-laying preference for 5% AA. Two representative lines, GAL44-120 and GAL44-98, showed strongly reduced oviposition preference at 30 °C in the presence of UAS-ShiP2 (Figs. S4 and S6A). Meanwhile, positional aversion to 5% AA was unaffected in experimental and control flies (Figs. S4 and S6A), providing evidence for dissociation between the competing behavioral choices toward AA. Expression of GAL4 in both the GAL44-120 and GAL44-98 lines, visualized with a UAS-GFP transgene (25), was preferentially found in the MB, some lateral neurons (LNs), and a few scattered cells in the brain (Figs. S8 and S6B). Assays conducted with pdf-GAL4/UAS-ShiP2 flies, which express GAL4 specifically in LNs, did not affect egg laying or positional responses (Fig. S7). Furthermore, we did not detect GFP expression in olfactory and gustatory neurons of GAL44-120 and GAL44-98 lines. Thus, the observed phenotypes were not due to silencing of LNs or sensory systems.

We also identified 4 lines with highly specific expression in the ellipsoid body (EB) ring neurons that exhibited disrupted positional aversion to 5% AA. Two representative lines, GAL44-67 and GAL44-72, showed reductions in positional aversion to 5% AA in the presence of UAS-ShiP2 at 30 °C (Figs. S5C and S6C) when compared with the singly transgenic controls. Egg-laying preference in the experimental flies was essentially unchanged compared with the respective singly transgenic controls. Egg-laying and positional preferences at the permissive (23 °C) and nonpermissive (30 °C) temperatures. (Fig. 3)

**Fig. 5.** Effect of silencing specific neuronal subsets with Shibire. OI and PI values for UAS-ShiP2+ and GAL4+/+ controls, and GAL4/UAS-ShiP2 experimental females at permissive (23 °C) and nonpermissive (30 °C) temperatures. (A) GAL44/UAS-ShiP2 exhibited reduced egg-laying preference for 5% AA at 30 °C, while maintaining normal positional aversion. (B) Brain GAL4 expression of GAL44-120, visualized by crossing to UAS-CDB.GFP, revealed strong expression in the mushroom body (MB) and a few lateral neurons (LNs). (C) GAL44-67/UAS-ShiP2 exhibited strong reduction in positional aversion at 23 °C and 30 °C, while maintaining normal egg-laying attraction. (D) GAL44-67 drives strong expression in neurons that project to the ellipsoid body ring (EB). The locations of cell bodies (cb), dendrites (d), and axonal terminals (t) are indicated. (A and C, *P < 0.05; **P < 0.01; ***P < 0.001 by 1-way ANOVA with Bonferroni’s post-test for comparisons between columns within the 23 °C or 30 °C groups; n ≥ 8). (B and D: green = UAS-CDB.GFP, red = neurolip marker nc82).

**Fig. 6.** Models for the interaction between egg-laying attraction and positional repulsion to AA. The gustatory (GS) and olfactory (OS) systems simultaneously detect input from a single compound, AA. Both sensory systems relay the signals to higher order centers of their respective circuits for processing and subsequent execution of motor programs (MS = motor systems) leading to oviposition preference (OP) or positional avoidance (PA). Competition between behavioral drives could occur: (1) in the female brain where neurons of the 2 pathways interact to simultaneously evaluate competing signals, such that either oviposition preference (OP) or positional avoidance (PA) is selected before motor program execution; (2) at the behavioral output where program execution inhibits the other pathway; (3) a combination of both central integration and behavioral output competition; (4) and (5) as directional inhibitory interactions between either the gustatory (4) or olfactory (5) processing circuits and corresponding behavioral outputs. Red intersection lines represent negative interactions.

(Figs. 5C and S6C). GAL44-67 and GAL44-72 lines express GAL4 primarily in the EB ring neurons, (Figs. 5D and S6D), peripheral sensory structures revealed no GFP expression. Of note, females showed increased positional aversion to 5% AA at 30 °C (Figs. 5 and S6), likely due to enhanced olfactory input caused by higher volatility of AA at 30 °C; this effect was consistent across all genotypes and thus, did not confound data interpretation. GAL44-67 and GAL44-72 also showed disrupted positional aversion in the presence of UAS-ShiP2 at 23 °C (Figs. 5C and S6C), an effect likely caused by residual function of the UAS-ShiP2 transgene in neurons that are particularly sensitive to synaptic silencing (26). We were unable to determine if the disruption in positional aversion seen upon silencing EB neurons was associated with an increase in egg-laying preference, as the latter was nearly maximal at 5% AA. Attempts to carry out these tests at lower AA concentrations were unsuccessful, as changes in positional responses were too subtle for definitive conclusions.

To further investigate whether the MB and EB function in separate or interconnected pathways, we simultaneously silenced both regions in "double-GAL4" flies carrying GAL44-120, GAL44-67 and UAS-ShiP2. Cross-talk between the 2 circuits could manifest as nonadditive (synergistic or epistatic) effects on the behavioral choices. Compared with the respective single GAL4/UAS-ShiP2 transgenes, double-GAL4 females showed disruptions of oviposition and positional preference that were essentially the same as those seen with the independently silencing GAL4 lines (Fig. S8), which suggests the MB and EB function in largely separate pathways to affect egg-laying attraction and positional repulsion to 5% AA, respectively.

**Discussion**

Our data provide a neurobehavioral model in which AA, a single ecologically relevant input, is detected by separate sensory systems to generate 2 distinct behavioral outputs: gustatory-based egg-laying attraction and olfactory-based positional repulsion (Fig. 6). We postulate *D. melanogaster* has an innate positional repulsion to the smell of AA. However, when needing to lay eggs, the attraction for AA overrides this positional repulsion, thereby allowing females to deposit their eggs on...
AA-containing food. Other studies have revealed opposing behavioral responses to a single compound; when detected as carbonation by the gustatory system, CO₂ is attractive (27), but when detected as an odorant, it is aversive (28). However, our experimental setup is unique in that opposing behavioral responses to a single stimulus (AA) are concurrently induced and assayed, affording direct observation of the competition between the 2 behavioral drives.

Several models can be invoked to explain the data surrounding these competing drives. In 1 extreme model, information gathered by the olfactory and gustatory systems would be processed by a set of common neurons, where concurrent evaluation of sensory input from both pathways would result in the selection of either repulsion or attraction before a final motor program for each behavior is executed (Fig. 6, no. 1). This model requires that these neurons simultaneously integrate sensory inputs to drive either egg-laying or positional behaviors. In the alternative extreme model, gustatory and olfactory signals would be independently processed by parallel neural circuits, such that attraction and repulsion only compete at the behavioral output level, after motor-program selection, since a female fly can only be in 1 place at a given time (Fig. 6, no. 2). Combinatorial models invoking both central integration and competition of behavioral outputs are also possible (Fig. 6, no. 3–5).

Models that involve competition through central integration imply that signals converge on common neurons in higher brain centers, and therefore, silencing these neurons would be expected to disrupt both egg-laying attraction and positional repulsion. In our limited Shi⁰ screen, we did not identify such a region. However, we did find higher-order structures that regulate each individual preference pathway. The MB appears to mediate taste-based attraction to AA for egg-laying purposes. Given the role of the MB in olfactory learning and memory (29, 30), it was surprising that it regulates taste-based behavior in our paradigm. However, a neural connection between the suboesophageal ganglion, which receives gustatory input, and the MB has been described recently in honey bees (31), providing a possible neuroanatomical link. Meanwhile, the EB (likely the R1 and R4 ring neurons) plays a role in olfactory-based positional repulsion to AA. Our data are consistent with studies showing the EB plays a role in olfactory-related tasks (30, 31) and spatial memory (32).

Exactly how and where the MB and EB function in the neural circuits that regulate AA responses remains to be determined. However, the results obtained with MB and/or EB silencing allow us to draw important conclusions regarding the models presented in Fig. 6. Experiments with poxn flies showed that abrogating gustatory input upstream of potential central integration in the brain not only impaired egg-laying preference for AA, but also caused a concomitant enhancement of positional aversion. In contrast, synaptic silencing of the MB, while also causing a robust decrease of egg-laying preference, did not result in a compensatory increase in positional aversion. These data 1) argue against competition of behavioral outputs as the sole mechanism responsible for selection between behavioral responses, and therefore, some cross-talk between olfactory and gustatory inputs must occur centrally, and 2) strongly suggests that the MB functions downstream of such cross-talk, after a positional response has been chosen, as its silencing affects only the motor program involved in egg-laying preference without altering positional aversion. With regards to the EB, our double-GAL4 experiments (Fig. S8) revealed an additive effect, leading us to hypothesize that the EB functions in parallel to the MB to control the motor program involved in positional aversion. Thus, potential cross-talk between the 2 circuits could also occur upstream from the EB.

Our data clearly show that disrupting peripheral sensory input causes compensatory shifts in egg-laying attraction and positional aversion (Figs. 3, 4, and S5). Thus, despite evidence for central integration, competition between behavioral outputs contributes to the overall response of flies when choosing a substrate for oviposition. Such competition arises from a logistical issue; flies lay equal numbers of eggs on regular food or food supplemented with 5% AA when not given a choice, but when provided with the choice of both oviposition substrates, they lay approximately 90% of their eggs on AA-containing food. Since laying an egg takes time (8), and females cannot be in 2 places at the same time, the OI and PI values must be at least partially correlated. Thus, our data supports a model where both central integration and competition of behavioral outputs mediate the choice-like behavior elicited when females encounter different oviposition substrate options (Fig. 6, no. 3).

We suggest that our paradigm can be used as a simple model for choice-like behavior in D. melanogaster. Supporting this possibility, a recent study by Yang et al. (8) employs a different paradigm for simple decision-making, in which females use their gustatory system to evaluate bitter and sweet egg-laying substrates. Our model differs in that it uses a single compound to stimulate competing drives via 2 distinct sensory modalities. Both systems provide powerful new paradigms to study the molecular and neural bases of simple decision-making in D. melanogaster.

Methods

Fly Stocks. Behavioral analysis and white rabbit (whir+) experiments were performed in w¹¹¹⁸ Berlin genetic background. The poxn⁰M22-85 lines used were a mixed w Berlin background, in which flies contained the original poxn⁰M22-85 second chromosome, but all other chromosomes were from the w¹¹¹⁸ Berlin strain. UAS-Shi⁰ transgenic flies contained 2 insertions of the transgene in a w¹¹¹⁸ Canton S background. Unless otherwise noted, flies were reared in constant light, 25 °C, 70% humidity on cornmeal/molasses/yeast food.

Two-Choice Oviposition and Positional Assays. The 2-choice apparatus was assembled using plastic 6-oz round-bottom bottles with the base cut off and replaced with a transparent 60-mm Petri dish lid. Food substrate was made by mixing the appropriate volume of experimental compound or H₂O into molten fly food at temperatures below the boiling point. Two-choice dishes were made by dividing a 35-mm Petri dish lid with a razor blade, and pouring 2 samples of food-substrate into each half (see SI Methods for detailed description). For each test, 15–20 recently-eclosed females were collected and mated for 2–3 days. Flies were gently knocked into the assay bottle without anesthesia to eliminate CO₂-based artifacts, and allowed to sample for 3 h. To determine oviposition preference, the amount of eggs on each half of the 2-choice dish was counted, and an oviposition index (OI): OI = (no. of eggs laid on experimental food – no. of eggs laid on control food) / no. of total eggs laid. For positional preference, the number of flies on each half of the dish was counted at 15-min intervals for 3 h. The number of flies was totaled, averaged, and a position index (PI) was calculated: PI = (flies on experimental food – flies on control food) / (flies on experimental food + flies on control food). Variants of this 2-choice setup, including the stripe assay and single-fly tracking are described in SI Methods.

Feeding Assay. To assay feeding preferences, the food mixing protocol was modified such that either Erioglaucine (FD&C Blue #1) or Fast Green FCF dye (Green #3) was mixed into the experimental (5% AA) or control (5% H₂O) food. Females were allowed to feed for 4-h, after which they were frozen, homogenized, and extracts centrifuged to remove insoluble material. Dyes in the supernatant were separated by thin layer chromatography (see SI Methods for detailed protocol).

Generation of Food of Different Acidity. We empirically measured the concentrations of AA, HCl, and H₂SO₄ that yielded food-substrate mixtures with equivalent pH values between 2.0 and 4.5 by using pH indicator strips. To verify these measurements, hardened food was re-heated, diluted 1:10 in distilled water, and the pH of the resulting solution was measured using a pH meter. Acid concentrations yielding equivalent pH values are listed in Table S2.
**Surgeries.** Females were anesthetized with CO₂, and the third antennal segment was removed with a set of sharp forceps. Flies recovered for 2 days before testing.

**Brain Regions Involved in Egg-Laying and Positional Preference.** We selected 58 lines with GAL4 expression in restricted neuronal subsets of the adult fly brain (Table S1). GAL4 lines were crossed to flies carrying UAS-Shi transgenes. GAL4/UAS-Shi, GAL4+, and UAS Shii+ females were placed at room temperature (23 °C) or in an incubator (30 °C) and allowed to equilibrate for 30 min, after which the number of flies on each half of the dish was counted at 10-min intervals. After 8 time points (t = 70 min), both the 23 °C and 30 °C experiments were moved to the dark for the remainder of the assay for optimal egg laying.

**Immunohistochemistry.** GAL4/UAS-CD8:GFP fly brains were immunostained with an antibody against GFP and nc82 and imaged by using a Leica confocal microscope (see SI Methods for details).

**Statistics.** All statistical analyses were performed using GraphPad Prism, Version 4.0 (GraphPad Software, Inc.). Statistics were performed independently on oviposition preference data and position preference data. Error bars in figures, mean ± standard error of the mean (S.E.M).

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