

Multimodal Behavioral Phenotyping of Stress-Induced Depression-Like States in *Drosophila melanogaster*

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Abstract

Metabolic, neurodegenerative, and stress-related disorders are frequently accompanied by altered locomotion, impaired decision-making, and reduced behavioral flexibility. However, accessible multimodal behavioral frameworks for quantifying these phenotypes in *Drosophila melanogaster* remain limited. Here, a protocol integrating forced swim exposure, Y-maze turning behavior and handedness, phototaxis, and activity assessment in the FlyVac system, open-field exploration, and long-term locomotor monitoring using the *Drosophila* Activity Monitor is presented. These complementary assays capture multiple dimensions of behavior, including motor output, motivation, decision structure, and behavioral variability, as functional readouts of neural and metabolic states. The pipeline is scalable, reproducible, and adaptable to pharmacological, genetic, and environmental manipulations, providing a versatile framework for detecting stress-, metabolic-, and neurodegeneration-related behavioral phenotypes in *Drosophila*.

Introduction

Behavioral alterations such as changes in locomotion, decision-making, and exploratory activity are widely used as organism-level indicators of neural and physiological state. Across taxa, neural dysfunction often manifests as altered movement structure, reduced exploratory motivation, impaired sensory-guided decision behavior, and changes in behavioral predictability. Because these organism-level outputs integrate neural, metabolic, and physiological state, they provide scalable readouts for detecting disease-relevant phenotypes. However, despite the extensive use of *Drosophila melanogaster* in molecular genetics and neuroscience, standardized multimodal behavioral frameworks

that simultaneously capture locomotor performance, decision structure, and behavioral variability remain limited.

Model organisms are essential for studying mechanisms underlying stress-related and neuropsychiatric phenotypes because controlled experimental manipulation is often impossible in humans. Stress-related behavioral syndromes occur across many taxa, including insects, and involve conserved neuromodulatory systems, such as serotonin and dopamine, as well as metabolic signaling pathways. The fruit fly has therefore emerged as a powerful system for linking molecular dysfunction to organism-level behavioral outcomes due to its short generation time, genetic tractability, and conservation of disease-relevant pathways^{1,2}.

At the same time, many behavioral assays in *Drosophila* are typically applied in isolation, making it difficult to distinguish between general sickness effects, motor impairment, motivational changes, and decision-level behavioral alterations. Moreover, mean behavioral measures alone may obscure biologically meaningful phenotypes, as neuromodulatory and developmental perturbations often alter behavioral variability rather than average behavioral output. A multimodal behavioral approach combining locomotor, exploratory, sensory, and decision-making assays is therefore necessary to generate robust organism-level phenotypic signatures³.

Here, a multimodal behavioral pipeline integrating forced swim exposure^{4,5}, Y-maze turning behavior and handedness^{6,7,8,9}, phototaxis and activity assessment using the FlyVac system^{10,11}, open-field exploration¹², and long-term locomotor monitoring using the *Drosophila* Activity Monitor^{5,13} is presented (**Figure 1**). Each assay in this framework captures a distinct behavioral dimension relevant to stress-related phenotypes. The forced swim test measures passive coping behavior, where an earlier transition to immobility reflects reduced motivation to sustain escape responses. The open-field and DAM assays quantify general locomotor activity and circadian structure, providing baseline measures of motor output and arousal. The Y-maze assay captures decision-making structure through sequences of left–right choices, allowing estimation of both turning bias (lateralization) and behavioral variability as a measure of predictability. Finally, the FlyVac assay evaluates sensory-guided decision-making and approach–avoidance behavior through repeated phototactic choices. Together, these assays provide complementary readouts that distinguish changes in activity, motivation, and decision-level behavior.

This protocol captures complementary aspects of motor output, motivation, decision structure, and behavioral variability, providing a scalable framework for detecting stress-, metabolic-, and neurodegeneration-related behavioral phenotypes in *D. melanogaster*¹⁴. The primary aim of this study is to establish a multimodal behavioral phenotyping framework that integrates complementary assays to capture multiple dimensions of stress-induced behavioral change. The stress paradigm is used here as a standardized perturbation to demonstrate the framework's sensitivity and applicability.

Protocol

The forced swim assay was conducted in accordance with the principles of the 3Rs (Replacement, Reduction, and Refinement). Replacement was achieved by using *Drosophila melanogaster* as an invertebrate model, thereby avoiding vertebrate forced swim paradigms that are increasingly restricted for ethical reasons. Reduction was supported by the assay's high-

throughput nature, which enables robust behavioral inference from relatively small cohorts while minimizing animal use. Refinement was addressed by limiting swim exposure to 2 min, a duration that prevents exhaustion or drowning and allows full recovery of normal locomotor behavior immediately after testing. Together, these considerations ensure that the assay provides meaningful behavioral data while minimizing potential distress and maximizing ethical acceptability, including in educational settings.

1. Induction of a depression-like state in *Drosophila* (vibration stress protocol)

1. Collect male *D. melanogaster* within 24 h of eclosion under light CO₂ anesthesia. All the details are mentioned in the **Table of Materials**.
2. Place flies in groups of 10 into polypropylene or acrylic vials (95 mm length × 25 mm inner diameter) sealed with cotton plugs.
3. Expose flies to mechanical vibration (300 Hz) using a vibration platform.
4. Apply repeated cycles consisting of 45 min vibration followed by 15 min recovery in vials containing standard food.
5. Continue vibration cycles for a total of 6 h per day.
6. Repeat the stress protocol for 3 consecutive days.
7. Maintain control flies under identical housing conditions without vibration exposure.

NOTE: In the present protocol, only male flies were used to reduce variability associated with sex-specific physiological and behavioral differences, including reproductive state and hormonal influences. This standardization facilitates the detection of stress-induced behavioral effects in a controlled setting. However, the protocol is fully applicable to female flies, and sex-specific responses represent an important area for future investigation. Given that sensitivity to stress-related and depression-like phenotypes can vary with sex and age across taxa, including *Drosophila*, extending this framework to different demographic groups may provide additional biological insight. This paradigm is adapted from established uncontrollable vibration stress models that induce motivational and activity alterations in flies.

2. Forced swim test in *Drosophila melanogaster*

CAUTION: Sodium dodecyl sulfate (SDS) is an irritant. Wear appropriate personal protective equipment (e.g., gloves and eye protection) when preparing and handling SDS solutions. Avoid skin and eye contact. Dispose of SDS-containing waste in accordance with institutional chemical safety guidelines.

1. Collect adult flies within 24 h of eclosion and maintain them under constant light and controlled environmental conditions.
2. Prepare multi-well chamber slides and fill each well with ~2 mL of 0.08% SDS solution at room temperature.
3. Transfer a single fly into each well immediately before recording. No anesthesia is required.
4. Record behavior using an overhead camera for 2 min to quantify latency to first immobility.
5. Quantify latency to first immobility.
6. Remove flies after recording and place them on absorbent paper to confirm recovery of normal locomotion.

NOTE: Immobility is defined as cessation of active escape movements while maintaining surface position. To record additional parameters such as total immobility duration and number of immobility bouts, longer recording durations (e.g., 3 min) are needed. This assay reflects passive coping behavior; reduced latency to immobility is interpreted as decreased motivation to sustain escape responses. Variability in latency to immobility / total immobility across flies. This assay may induce transient stress or fatigue. When used in longitudinal designs, perform it prior to other assays and allow sufficient recovery (e.g., ≥24 h) before subsequent testing.

3. Long-term locomotor activity monitoring using the *Drosophila* Activity Monitor (DAM)

1. Anesthetize fruit flies briefly (5 s) using CO₂ for sorting.
2. Place individual flies into glass DAM tubes containing food and cotton plugs¹⁷.
3. Insert tubes into the *Drosophila* Activity Monitor system.
4. Record locomotor activity continuously (48 h) under controlled temperature and light–dark conditions.
5. Store beam-break counts using acquisition software.
6. Bin activity data into defined time intervals (e.g., 1–10 min).
7. Exclude flies that die or show no activity after acclimation.

NOTE: Continuous locomotor monitoring over a 48-hour period is sufficient to capture circadian activity patterns. **DAM:** variability in total distance, velocity, and bout structure across individuals.

4. Open-field locomotor assay

1. Briefly (5 s) anesthetize fruit flies using CO₂.
2. Transfer individual flies into rectangular 25 x 75 mm arenas (one fly per arena).

3. Allow flies to acclimate (20 min) to the arena environment prior to recording under constant, low-intensity diffuse white light.

4. Record behavior under constant illumination using an automated video-tracking system.

5. Analyze trajectories using tracking software to extract:

Total distance moved

Average locomotor speed

Acceleration and turning metrics

NOTE: This assay provides a baseline for interpreting stress-related behavioral changes. The acclimation period minimizes handling-induced stress and allows flies to habituate to the arena, ensuring that recorded locomotor activity reflects baseline behavior. Constant illumination was used to reduce variability associated with circadian fluctuations and to ensure consistent behavioral conditions across trials. Trajectories can be extracted using automated tracking software such as EthoVision XT (Noldus Information Technology), or open-source alternatives including Ctrax, idTracker, and TrackMate (ImageJ/Fiji), depending on availability and user preference. **Open-field assay:** variability in total distance, velocity, and bout structure across individuals. To reduce order effects, standardize acclimation and lighting conditions across sessions. When multiple assays are conducted on the same individuals, keep arena exposure durations consistent.

5. Y-maze turning behavior and behavioral variability assay

1. Briefly anesthetize (5 s) fruit flies using CO₂.
2. Place individual flies into Y-maze chambers^{8,9}. Mazes were cut into 1.6 mm-thick black acrylic using a laser engraver. We placed flies in an array of 95 individual mazes, each consisting of three symmetrical arms (12 mm long and 3.3 mm wide).
3. Allow flies to explore freely for extended recording periods (20 min).
4. Record turning decisions (left or right) during exploratory locomotion (1 h).
5. Compute individual turning bias as the proportion of right turns. Calculate relevant metrics such as velocity, acceleration, motion without movement, time-based variations in activity^{12,15}.
6. Quantify between-fly behavioral variability using the median absolute deviation (MAD) of turning bias.

NOTE: This assay measures locomotor handedness and behavioral predictability independent of overall activity levels.

Turning sequences capture decision structure; turning bias reflects lateralization, while variability across individuals reflects behavioral predictability. *bias* = proportion of right turns (per fly); *variability* = dispersion (MAD_n) of bias across flies. For repeated testing, consider counterbalancing assay order across individuals or cohorts to control for sequence effects.

6. Phototactic choices: activity monitoring in the FlyVac apparatus

1. Load individual flies into FlyVac chambers^{6,11} without using CO_2 anesthesia.
2. Allow acclimation (10 min) before initiating trials.
3. Present repeated binary light–dark choices to each fly.
4. Record choices across 40 trials using automated detection.
5. Calculate light-choice probability per fly and the average time between light-choices as a measure of activity.
6. Quantify behavioral variability across individuals using MAD .

NOTE: The FlyVac system enables simultaneous assessment of mean decision bias and inter-individual variability. The behavioral assays described in this protocol are modular and can be conducted in different sequences depending on the experimental design. However, to minimize potential carry-over effects, performing the forced swim test prior to other assays is recommended. To reduce order effects, standardize acclimation and lighting conditions across sessions.

7. Data analysis

NOTE: Behavioral analyses were performed using individual flies as the unit of inference unless otherwise stated.

1. Forced swim test: Analyze the video recordings manually or using behavioral scoring software.
NOTE: Immobility was defined as the absence of active escape movements while the fly remained afloat. The following parameters were extracted per fly: latency to first immobility (s). Group comparisons were conducted using non-parametric statistical tests due to the non-normal distribution of behavioral measures.
2. Long-term locomotor activity (DAM): Record beam-break counts by the Drosophila Activity Monitor. Export and bin into 10 min intervals.

NOTE: For each fly, total activity counts and circadian activity patterns were calculated. These patterns can be calculated in R, Python, or Excel—whichever is available. Flies exhibiting inactivity due to mortality or technical artifacts were excluded prior to analysis. The assay lasted 48 h.

3. Open-field locomotor assay: Process the video-tracking data using automated tracking software. Import the video files into EthoVision XT. Perform tracking using automated detection with contrast-based thresholding.

NOTE: Trajectories were smoothed using the default filtering algorithm. Per-fly locomotor parameters (distance, velocity, acceleration) were exported as CSV files for downstream analysis. Per-fly locomotor parameters included: total distance moved (mm), accelerations (mm/s^2). Summary statistics were calculated at the individual level prior to group comparison. The duration of this test was 1 h for each group.

4. Y-maze turning behavior: Extract the turning decisions from recorded trajectories, classify as left or right, and export as binary sequences (left/right) for each individual.

NOTE: For each fly, the turning bias was calculated as the proportion of right turns across all recorded decisions. Between-fly behavioral variability was quantified using the scaled median absolute deviation ($MAD_n = 1.4826 \times MAD$) of turning bias. The duration of this test was 1 h for each group.

5. FlyVac phototactic choices: Record binary light–dark choices automatically. For each fly, calculate the light-choice probability (LCP) as the proportion of trials in which the light arm was selected. Express the phototactic bias as the mean of binary trial outcomes (light = +1, dark = -1).

NOTE: Between-fly variability was quantified using MAD_n of individual phototaxis indices.

6. Statistical analysis: Assess the group differences in behavioral measures using permutation-based tests with 10,000 label randomizations.

NOTE: To compare variability, permutation tests were performed on the absolute differences in MAD_n between groups. Confidence intervals for MAD_n estimates were obtained using bootstrap resampling. A two-tailed unpaired t-test was used to compare the Distance moved by flies (mm) and Maximum acceleration (mm/s^2). For data visualization, violin plots (primarily to show density, concentration, and skewness), box plots, and column charts were used. Statistical significance was evaluated at $\alpha = 0.05$.

Results

Application of the vibration stress protocol produced measurable behavioral alterations across multiple assays (**Figure 1**) designed to capture activity, coping behavior, and decision-making in *D. melanogaster*.

Depression-like induction

Flies exposed to repeated mechanical vibration (**Figure 1A**) displayed reduced exploratory activity and altered behavioral responses compared to unstressed controls. These changes were

stable across subsequent behavioral assays, indicating that the vibration paradigm effectively induced a persistent depression-like state.

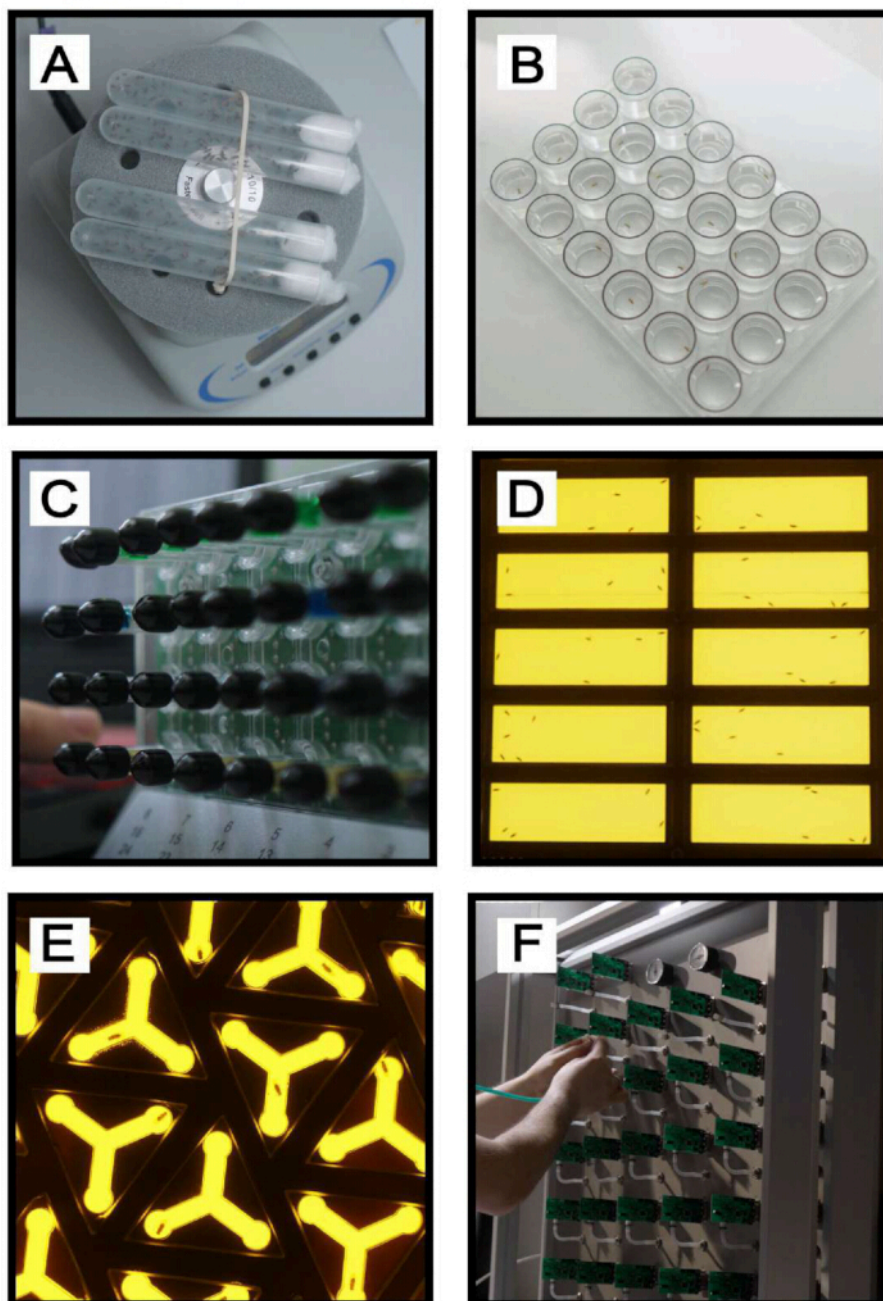


Figure 1: Representative experimental setups for behavioral assays. (A) Stress induction apparatus. (B) Forced swim test (FST). (C) Drosophila Activity Monitor. (DAM) system. (D) Open field test. (E) Y-maze test. (F) FlyVac apparatus. [Please click here to view a larger version of this figure.](#)

Forced swim test

During the forced swim assay (**Figure 1B**), control flies exhibited sustained escape movements before transitioning to passive floating behavior. In contrast, stressed flies typically adopted immobility more rapidly and showed prolonged passive episodes

(two-tailed Mann-Whitney U-test showed that the distributions of the two groups were significantly different: $U = 146$, $n_1 = n_2 = 25$, $P = 0.0032$; **Figure 2**). Representative recordings demonstrate clear behavioral transitions from active swimming to immobility, enabling reliable quantification of latency to immobility, total

immobility duration, and bout structure. The whiskers show the 10th to 90th percentile.

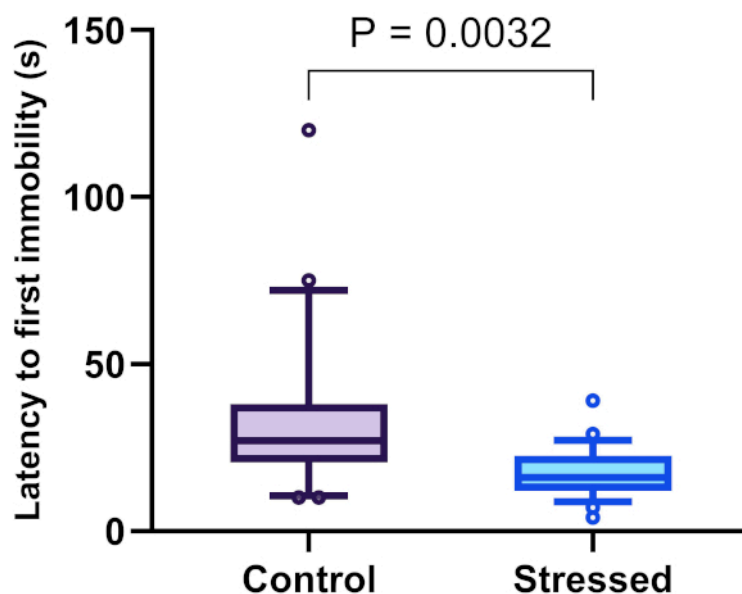


Figure 2: Differences between the control and stressed groups in time to reach the first immobility in the FST. Error bars show the 10–90 percentiles. [Please click here to view a larger version of this figure.](#)

Long-term locomotor activity monitoring (DAM)

Continuous DAM recordings (**Figure 1C**) revealed distinct activity patterns between groups: the flies of the control group were significantly more active than stressed flies (two-tailed Mann-

Whitney U-test showed that the distributions of the two groups were significantly different: $U = 72$, $n_1 = n_2 = 16$, $P = 0.0395$) (**Figure 3**). Error bars are $\pm$ SD.

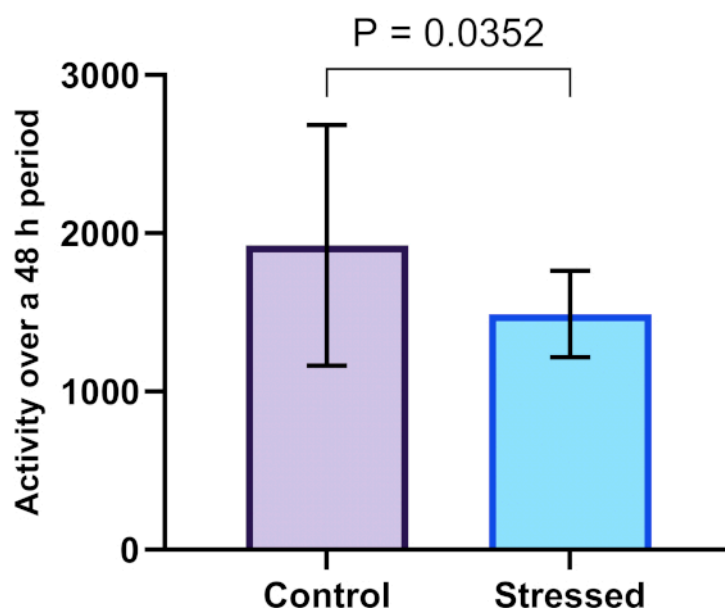


Figure 3: Total beam breaks over 48 h of the flies in the control and the stressed fly group. Error bars are $\pm$ SD. [Please click here to view a larger version of this figure.](#)

Open-field locomotor assay

In the open-field arena (**Figure 1D**), control flies ($n = 82$) explored the environment extensively, producing trajectories characterized by sustained locomotion and frequent directional changes. Stressed flies ($n = 88$) often exhibited reduced displacement and slower movement: the difference between the control group and stressed flies in the values of the total distance moved in 1 h (two-tailed unpaired t -test, $P < 0.0001$; **Figure 4A**) and

maximum acceleration (**Figure 4B**) was significantly different. Although control flies traveled greater distances than stressed flies (**Figure 4A**), the value of maximum acceleration was higher in the stressed fly group (mean $\pm$ SD: 158.9 ± 141.4 vs 206.6 ± 119.7 ; two-tailed unpaired t -test, $P = 0.0202$; **Figure 4B**). The solid line within the violin plot indicates the median, whilst the dotted line represents the 25th–75th percentile; the boundaries indicate the kernel density estimate range.

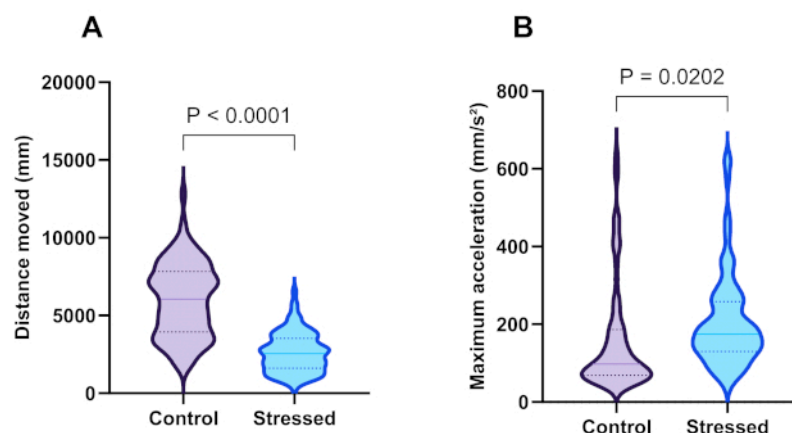


Figure 4: Differences in total distances. (A) Differences in total distances traveled in 1 h in the open field locomotor test. (B) maximum acceleration of control and stressed fruit flies in the open field locomotor test. The solid line in the violin plot indicates the median, whilst the dotted line represents the 25th–75th percentile; the boundaries indicate the range of the kernel density estimate. [Please click here to view a larger version of this figure.](#)

Y-maze turning behavior and behavioral variability

In the Y-maze assay (**Figure 1D**), individual flies generated long sequences of left and right turning decisions. While both groups displayed stable turning biases at the individual level, group comparisons showed dissimilarities in distances traveled and similar dispersion of turning preferences. The Y-maze test provided an opportunity to assess flies' total distance moved, maximum acceleration, and turning behavior variability. Control flies ($n = 59$) covered significantly longer distances in 1 h than stressed flies ($n = 60$) (**Figure 5A**) (two-tailed unpaired t -test, $P =$

0.0037; mean $\pm$ SD: 2321 ± 970.8 vs 1802 ± 943.4). Interestingly, stressed flies reached higher maximum acceleration than control flies (two-tailed unpaired t -test $P = 0.0041$, mean $\pm$ SD: 192.9 ± 160.1 vs 111.1 ± 143.7) (**Figure 5B**). However, there was no evidence for a group difference in turning bias ($P = 0.8733$, **Figure 5C**). Thus, depression induction did not measurably alter between-individual variability in turning bias in the Y-maze assay. The solid line within the violin plot indicates the median, whilst the dotted line represents the 25th–75th percentile; the boundaries indicate the kernel density estimate range.

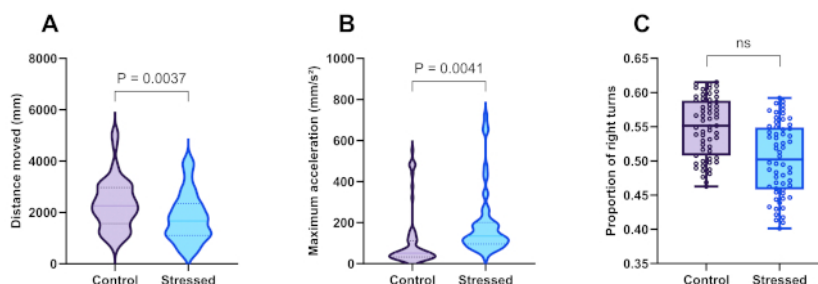


Figure 5: Y-maze: (A) total distance moved, (B) maximum acceleration, and (C) turning bias measured as the proportion of right turns. Whiskers extend to the absolute minimum and maximum data points. The solid line within the violin plot indicates the median, whilst the dotted line represents the 25th–75th percentile; the boundaries indicate the kernel density estimate range. [Please click here to view a larger version of this figure.](#)

FlyVac phototactic choices

Repeated binary phototactic trials in the FlyVac apparatus (**Figure 1F**) revealed stable individual choice patterns. Control flies ($n = 57$) typically demonstrated a consistent preference for light, whereas stressed flies ($n = 61$) showed a shift toward increased dark choices. Representative trial sequences illustrate

individual decision trajectories and highlight the capacity of the FlyVac system to capture both mean phototactic bias and inter-individual variability. The groups did not differ in the number of turns ($P = 0.2315$, **Figure 6A**). However, a confirmatory fly-level permutation test on the mean difference supported a lower LCP in stressed flies ($P < 0.0001$), consistent with a shift in approach–avoidance policy toward the dark arm (**Figure 6B**). While

depression induction strongly shifted mean phototactic choice, it

did not measurably alter between-fly variability under this protocol ($P = 0.239$, **Figure 6C**).

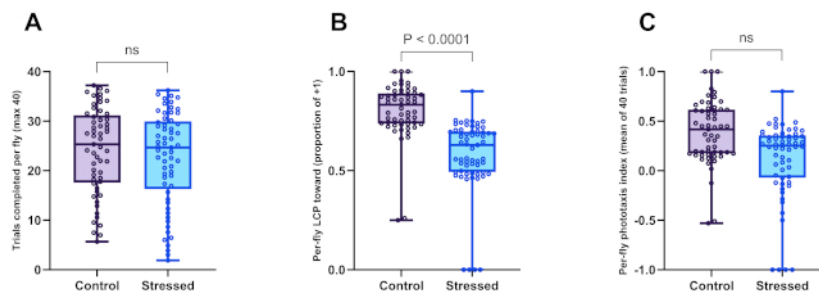


Figure 6: The results of the FlyVac test: (A) number of completed trials, (B) Light-choice probability, and (C) per-fly phototaxis index. Whiskers extend to the absolute minimum and maximum data points. [Please click here to view a larger version of this figure.](#)

Quantifying inter-individual variability. For each assay, individual-level metrics (e.g., phototaxis index, turning bias, locomotor measures) are computed per fly, and inter-individual variability is quantified as the dispersion of these values within a cohort tested under identical conditions. We use robust statistics such as the normalized median absolute deviation (MADn; $\text{MADn} = 1.4826 \times \text{MAD}$) to estimate between-fly variability while reducing sensitivity to outliers. Where appropriate, variability can also be summarized using variance or interquartile range.

Distinguishing variability from noise. Biological variability can be separated from measurement error and environmental noise using statistical models that incorporate replicate structure. For example, mixed-effects models can include individual identity as a random effect to estimate variance components (between-individual vs. residual), and intraclass correlation coefficients (ICC) can be derived to quantify repeatability. When repeated measures are available, variance partitioning can be used to separate within- and between-individual components. In addition, permutation tests or bootstrap resampling can assess whether observed between-individual dispersion exceeds that expected under random variation.

Longitudinal designs and carryover control. The framework is compatible with repeated-measures designs in which the same individuals are tracked across multiple assays. To minimize carryover effects, we recommend: (i) performing assays that may induce fatigue or stress (e.g., forced swim test) first; (ii) including recovery intervals between assays (e.g., ≥ 12 –24 h, depending on assay intensity); (iii) maintaining consistent environmental conditions (light, temperature, handling) across sessions; and (iv) randomizing or counterbalancing assay order when multiple sequences are feasible.

Discussion

The present protocol demonstrates a multimodal approach for inducing and quantifying depression-like behavioral states in *D. melanogaster*. Using a vibration-based stress paradigm combined with complementary behavioral assays, this study shows that depression-like induction can be detected across measures of coping behavior, locomotor activity, exploratory dynamics, decision-making, and behavioral variability¹⁶. The stress regime employed in this study serves as a practical tool to elicit measurable behavioral changes and to demonstrate the utility of the multimodal framework, rather than representing the primary focus of the protocol. This integrated framework highlights the value of combining traditional activity-based readouts with measures of behavioral individuality. The forced swim test results obtained in this study are consistent with previous work validating this assay as a measure of passive coping behavior in flies. Similar to earlier findings, stressed flies displayed altered swimming behavior characterized by increased immobility, supporting the reliability of vibration stress as an induction paradigm⁵. Together with earlier demonstrations of stress-induced behavioral changes in *Drosophila*, these findings reinforce the forced swim assay as a robust component of depression-related behavioral phenotyping¹⁸.

Long-term locomotor monitoring using the *Drosophila* Activity Monitor further confirmed the presence of stress-related behavioral alterations. Consistent with prior studies, stressed flies exhibited reduced activity levels compared to controls⁵. While DAM-based measurements provide valuable continuous activity readouts, our results suggest that they capture only part of the behavioral phenotype. Specifically, assays involving spatial exploration and decision-making revealed additional aspects of behavioral change that cannot be fully inferred from beam-break counts alone. The open-field locomotor assay and

the Y-maze paradigm extended the behavioral characterization by providing detailed spatial metrics. In agreement with earlier work, these assays revealed differences in movement trajectories, speed, and acceleration, offering a broader description of locomotor performance¹². Notably, acceleration measures appeared particularly sensitive to stress effects, supporting previous observations that dynamic movement parameters may better reflect motivational state than total distance alone⁵. This observation aligns with recent findings indicating that acceleration-based metrics can reveal subtle stress-induced behavioral alterations. Importantly, from a practical perspective, the Y-maze provides advantages in throughput, as multiple individuals can be monitored simultaneously using a single recording setup.

A distinctive contribution of the Y-maze assay lies in its ability to quantify behavioral variability. Turning bias represents a stable yet individually variable trait in flies, making it suitable for investigating individuality and predictability^{8,9}. In the present demonstration, no significant difference in variability between stressed and control flies was detected. However, the small sample size and binomial nature of turning decisions suggest that larger datasets are required to robustly evaluate variability effects, as highlighted in earlier large-scale behavioral studies. Future research integrating pharmacological manipulations may clarify the neurochemical mechanisms underlying variability in turning behavior. Although some assays in this pipeline provide partially overlapping locomotor metrics, they differ in the behavioral dimensions they emphasize. For example, the Y-maze assay captures decision structure and behavioral variability under constrained conditions, whereas the open-field assay provides a less constrained context for assessing exploratory behavior and spatial dynamics. These differences allow separation of locomotor performance from decision-level and motivational effects.

Importantly, the protocol is designed as a modular framework. Not all assays are required for every application, and researchers may select subsets of assays depending on the specific research question and available resources. While specialized systems such as DAM or FlyVac provide high-throughput, automated measurements, similar behavioral endpoints can be obtained using standard video-tracking approaches, thereby making the framework broadly accessible. The FlyVac phototactic paradigm further illustrated the importance of separating mean behavioral bias from variability. Consistent with established reports of strong photopositivity in Oregon R flies, control flies demonstrated a clear preference for light⁶. Stressed flies, however, exhibited a significant reduction in light-choice probability, indicating a shift in approach–avoidance decision-making. Notably, this shift occurred without detectable changes in between-fly variability, suggesting that depression-like induction may alter decision bias while preserving the structure of behavioral

individuality. This dissociation highlights the value of combining mean and dispersion metrics when characterizing affective-state manipulations.

In this framework, variability is not treated as noise but as a biologically meaningful trait reflecting differences in internal state, neural processing, and metabolic condition. By estimating dispersion across individuals under standardized conditions and, where possible, partitioning variance components, the protocol enables differentiation between stable individual differences and measurement or environmental noise. This is particularly relevant for detecting changes in behavioral organization and predictability under stress.

Taken together, the protocol demonstrates several methodological strengths. First, the vibration stress paradigm is non-invasive, reproducible, and compatible with high-throughput behavioral testing. Second, the combination of assays captures multiple dimensions of behavior, reducing the risk of over-interpreting results from a single metric. Third, the inclusion of variability-based measures provides an additional layer of analysis that may reveal effects not detectable through mean behavioral changes alone. Sex-specific differences in stress sensitivity, metabolism, and behavioral expression are well documented in *Drosophila* and other taxa. Although the present protocol was established using male flies to minimize baseline variability, extending this framework to females represents an important direction for future work and will be essential for fully characterizing sex-dependent responses.

The forced swim assay employed in this study was designed with explicit ethical considerations. Flies were exposed to the swim condition for only 2 min, a duration selected to avoid exhaustion or drowning while still allowing reliable quantification of coping behavior. Under these conditions, all individuals recovered normal locomotion immediately after testing, indicating that the procedure does not produce lasting harm. The short exposure time also reduces potential distress and supports the use of the assay in educational settings, where students can perform behavioral experiments without concerns about animal injury. More broadly, the use of *Drosophila melanogaster* as a model organism provides an ethically favorable alternative to vertebrate forced swim paradigms, which are increasingly restricted or discouraged in many countries. Thus, the present implementation combines methodological validity with refinement principles by minimizing exposure duration and replacing vertebrate models with an invertebrate system.

The multimodal protocol described here offers broad applicability for studies of stress, affective states, and behavioral individuality in *Drosophila*. Potential applications include screening of antidepressant compounds, investigation of gene–environment interactions, and exploration of neurobiological mechanisms underlying variability in behavior. The multimodal framework

described here can be readily combined with genetic or pharmacological manipulations, including knockdown or overexpression of candidate genes, to establish causal links between molecular pathways and behavioral phenotypes. Although the present study emphasizes behavioral phenotyping and face-valid outcomes, full validation of depression-like models also includes predictive and construct validity. In particular, pharmacological reversal of behavioral phenotypes represents an important test of predictive validity. The multimodal framework described here is well-suited for such applications and can be readily combined with antidepressant treatments or other interventions to assess the reversibility of stress-induced behavioral changes. The integration of reproducible stress induction with complementary behavioral assays provides a flexible platform for advancing translational behavioral neuroscience using invertebrate models. The multimodal protocol can be extended to longitudinal designs that track the same individuals across assays, enabling integrated assessment of activity, motivation, and decision-making. Careful control of assay order, recovery intervals, and environmental conditions is essential to minimize carryover effects. In cases where strong interference is expected, parallel cohorts can be used to isolate specific behavioral domains.

Although the present protocol demonstrates robust detection of stress-induced behavioral changes across multiple assays, formal quantification of within-individual repeatability and between-cohort reproducibility was not the primary objective of this study. Future work applying this framework could incorporate repeated-measures designs and multi-cohort comparisons to estimate reliability metrics such as intraclass correlation coefficients and variance components. The standardized and scalable nature of the protocol makes it well-suited for such analyses. The present protocol provides a reproducible and scalable framework for inducing and quantifying depression-like behavioral states in *D. melanogaster*. The vibration stress paradigm, combined with complementary behavioral assays—including the forced swim test, long-term locomotor monitoring, open-field exploration, Y-maze turning behavior, and FlyVac phototactic choices—enables multidimensional characterization of stress-induced behavioral changes. Importantly, this approach integrates measures of both central tendency (mean behavioral bias) and behavioral variability, allowing separation of motivational shifts from changes in individuality structure. The protocol is adaptable to pharmacological, genetic, and environmental manipulations and offers a versatile platform for studying affective-state regulation and behavioral phenotyping in invertebrate models.

While the multimodal protocol presented here enables comprehensive behavioral characterization of depression-like states in *Drosophila melanogaster*, several limitations should be considered. First, behavioral assays differ in their sensitivity to

locomotor suppression, which may confound the interpretation of motivational changes in certain contexts. Second, variability-based analyses require relatively large sample sizes to reliably detect subtle differences in dispersion across individuals. Third, the vibration stress paradigm represents one model of depression-like induction and may not capture all neurobiological aspects of affective disorders. Finally, environmental factors such as temperature, humidity, and handling procedures can influence behavioral outcomes and should be carefully controlled. Despite these limitations, the protocol provides a reproducible framework that can be combined with pharmacological, genetic, or environmental manipulations to strengthen mechanistic interpretation.

Disclosures

The authors declare no conflicts of interest.

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