**Quantifying between-individual variation using high-throughput phenotyping of behavioural traits in the fruit fly (*Drosophila melanogaster*)**

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**Introduction**

**Methods**

*Fly colony*

Our study used a large, outbred population of Canton-S wild-type fruit flies sourced from a stock maintained at the University of Sydney. The flies were under the 12h:12h photoperiod in a temperature-controlled room maintaining constant thermal conditions (25°C) and humidity of approximately 45-65%. The flies were kept on a commercially available food medium (…) that was replaced with new portions once or twice weekly. The colony was kept in a small plastic terrarium, and food was delivered in open bottles arranged on the bottom of the tank.

Before each test a sample of several dozens of flies was transferred into an empty, dry culture bottle and briefly submerged (for a period of 3-5 minutes) in a bucket full of ice to induce chilling coma. Following coma induction, the flies were transferred into their assay arenas (see the next section for detailed description) using aspirators. Before each test, the flies were allowed to return to full activity for approximately 15 minutes by leaving a loaded set of arenas in a lit area with temperature of 25°C. Each batch of tested flies consisted of individuals repeatedly tested in three different behavioural assays (see below). Transferring the flies between assays using different arena designs was achieved by briefly inducing chilling coma and aspirating the flies between arenas. At the end (following all tests) the flies were euthanised by leaving them overnight in a freezer (-25°C). the following day all individual insects were sexed, following established criteria.

In total, we have assayed 384 flies, arranged into 4 batches, 96 flies each.

*Behavioral assays*

We have employed three types of assays, each performed using a multi-well plexiglass or polystyrene plate able to hold between 15 and 48 flies (depending on the assay type).

*Locomotion trakcing.* In this assay, individual flies were loaded into 1 cm deep, round, transparent arenas arranged into a 48-well plate. For the purposes of this study, we have used 48-well cell-culture plates (NEST Biotechnology Co., Ltd.; China); the choice of this particular brand was dictated by nearly no gap space between each well’s rim and the lid covering whole plate, which prevents flies from escaping the wells and migrating to adjacent arenas. Locomotion of flies individually kept in the arenas was followed by recording their position (see the *Phenotyping units* section for technical details) in short intervals, which was then used to calculate the distance travelled by each fly in a set interval of time. In our assay the flies were tracked for 5 consecutive intervals, 10 minutes each.

*Habituation assay*. In this test we have used the established startle response of fruit flies in response to a brief light-off stimulus (Allen and Budenberg, 2021). The response is elicited by a short light-off pulse lasting 15 ms and can be measured as a sudden and very fast movement (often involving flight) of stimulated flies. The stimulus was delivered by an optogenic light-conducting plate mounted beneath the 48-well plate with experimental arenas. The device (part of the ZANTIKS MWP phenotyping system, see the next section for more details) consists of a plexiglass plate coupled with a set of several very bright LEDs. The diodes emit green light (530 nm) flooding the experimental arenas with over 7000 lux of light. The system is connected to the phenotyping unit and delivers stimuli in the form of brief light-off pulses.

The objective of our experiment was to study habituation to the light-off stimulus. Thus, in our assay we subjected the flies to three consecutive 15 ms light-off pulses, 1 second apart. The startle jump response (distance covered by each fly) was recorded within a 1 second interval following each light-off pulse.

*Y-maze tracking*. Third assay was performed to explore fruit flies’ behaviour in a simple 3-arm maze (Simonnet *et al.*, 2014; Cleal *et al.*, 2021). The test was performed in a different type of arena: flies were loaded into small plexiglass blocks with a y-shaped forking channel etched inside of them. The maze was covered by a sliding coverslip that allowed for easy loading and unloading of mazes with flies. Each experimental plate consisted of 15 such mazes (which means that – in order to match the mazes to flies tested in 48-well plates – we have discarded 3 random flies from each 48-well plate, before loading them to three y-maze plates hosting 45 flies in total). Tracking of flies’ behaviour in the mazes lasted 30 minutes, and apart from recording the time spent by flies in each of three maze arms we have also recorded each arm crossing event (i.e., a fly crossing from one maze arm to another, through the central “neutral zone”). The protocol we used was based on a comparative study looking at Y-maze exploration behaviour in mice, zebrafish and fruit flies (Cleal *et al.*, 2021).

*Phenotyping units*

All behavioural tests were performed using automated tracking units produced by Zantiks (Cambridge, UK). The units we employed were from the WMP series, suitable for tracking of small-sized animals such as small insects, crustaceans, fish larvae, etc. Each unit consists of a computer that controls its operation, an experimental chamber that can host experimental arenas (and can be connected to a temperature control unit, able to maintain internal chamber temperature in a narrow, set range) and a camera system able to track animal movement in arenas inserted to the chamber.

All of arenas used in our assays had the same format (i.e., dimensions of a standard ELISA multi-well plate) and were placed inside each unit on a raised stand (locomotion and Y-maze assays) or the designated optogenic stand (habituation assay). In order to be able to track the animals, the unit requires a correct definition of experimental arenas (a bitmap file mapping regions of the recorded image to specific experimental arenas) and a so called autoreference process that removes actual animal from the immobile background (thus allowing it to be traced using the actual experiment). The autoreference stage was programmed into each assay and lasted 10 minutes (locomotion and Y-maze assays) or 5 minutes (habituation assay) to make sure that within this interval each individual had moved in its arena.

Experimental procedures in the units are controlled by a scripting language (Zanscript) – scripts describing the three assays used in our study can be found in the paper’s GitHub repository (…) in the *Protocols* directory.

*Data analysis*

The habituation and locomotion data were used as they were, i.e., the respective responses (see above for details) were used in downstream procedures. Since the files produced by Zantiks units have a particular form (a header section with technical details, followed by actual data formatted according to the script run on the machine, and followed by a footer), data processing involved parsing each file to extract the most relevant information (e.g., experimental unit ID, assay ID, run date, formatted data matched with the numbers of experimental arenas). Parsing steps used in each assay type are presented in the GitHub repository (please see the *R* directory for a detailed RMarkdown document). Pre-processing of the habituation assay outputs was based on an earlier study applying the same test (Allen and Budenberg, 2021).

The Y-maze behavioural test outputs required more sophisticated processing. Our protocol is based on a modified analysis from Cleal *et al.* (2021). In brief, the analysis extracted all maze arm switches observed during the assay (i.e., walks between two maze arms, termed zone changes). The switches were then classified as left- (L) or right(R)-turns, and series of consecutive L/R-turns were assembled into triplets. We have then calculated the proportion of alternating (LRL or RLR) vs. sequential (LLL, RRR) vs. partial (RRL, LLR) movements in the total count of all possible maze explorations (which also include returning to the same zone).

Resulting response variables were analysed using linear mixed models with a gaussian (continuous variables) or Poisson (count variables) error distribution. Mixed models were fitted using the *lmer*/*glmer* fucntions in the *lme4* package in R. Each model contained fixed effects of sex and experiment round (if applicable – this variable was present only in experiments performed repeatedly, i.e., locomotion tracking and habituation assay). Batch ID was included as a random effect. Continuous variables were log-transformed wherever needed (based on the visual inspection of model residuals) and zero-centred.

**Results and Discussion**

Locomotion tracking