nature research

Corresponding author(s): Noah K. Whiteman

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☐	☒	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated
☐	☒	Clearly defined error bars *State explicitly what error bars represent (e.g. SD, SE, CI)*

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection	The StepOne™ Real-Time PCR System (ThermoFisher Scientific) was used for qPCR. The Agilent 1100 series HPLC unit (Agilent, Santa Clara CA) was used to detect ouabain in the sequestration assay.
Data analysis	Tree construction was performed with IQ-TREE (Trifinopoulos et al. 2016). Analysis of residue associations with feeding was performed with TraitRateProp (Karin et al. 2017), and the makeChronosCalib function of the ape package in R (Paradis et al., 2004). Coevolving sites analysis was performed with Spidermonkey/BGM in HyPhy (Poon et al. 2007). Ancestral sequence reconstruction was performed in HyPhy (Pupko et al. 2000; Pond et al. 2005). Feeding and sequestering ancestral state reconstruction was performed with the ace function of the ape package in R (Paradis et al., 2004). The tree and character states were visualized using Interactive Tree Of Life (iTOL) v3 (Letunic et al., 2016), and can be found here: https://itol.embl.de/tree/136152143114962156113638l. Origin-Pro 9.1G was used to determine the Hill plots and coefficients. Statistical analysis was performed using Prism 8 (Graphpad) or R (including some analyses based on code developed originally for MATLAB). Protein homology modeling and in silico mutagenesis was performed via Modeller (Sali and Blundell, 1993), PyMod2 (Janson et al., 2016), ClustalW and PyMOL (Schrödinger, 2015). Molecular docking simulations were performed with Autodock4 (Morris et al. 1998; Huey et a. 2007). Statistical analysis to assess the reproducibility of the mutational order was performed with a modified custom algorithm in MATLAB (Toprak et al., 2011) kindly provided ny Prof. Erdal Toprak. Please refer to Methods for details.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Raw data for all bioassays are provided in the associated Source data excel file of each Figure. Correspondence and requests for other materials should be addressed to N.K.W. (whiteman@berkeley.edu)

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	We performed preliminary experiments to assess variance and estimate sample sizes before conducting all bioassays, following sample sizes published for studies conducting identical or similar experiments, most of which were papers that included present co-authors. For feeding experiments, we relied on previously published studies using feeding experiments with ouabain and Drosophila. Although our sample sizes for feeding experiments on fly media were 10-200 flies per vial for the various feeding experiments, a sample size of N = 3 per vial with 3 biological replicate vials was used for the CAFE assays, and this was chosen because similar assays were reported in a paper published by us in which we used feeding experiments with ouabain and Drosophila mutants in order to assess survival (Groen et al., 2017, doi: 10.1016/j.ibmb.2016.12.008). For other feeding experiments sample sizes ranged from N = 10 flies per vial to 200 eggs per vial (an over-abundance was used because of variance in survival across knock-in lines at eclosion that we report in the manuscript), and were thus conservative. For enzyme assays using insect heads, we followed previously published sample sizes for the number of heads per biological replicate (Petschenka et al., 2013, doi: 10.1111/evo.12152), and similarly for enzyme assays using Sf9 cell lines (Dalla and Dobler, 2016 ,doi: 10.1111/evo.13077). For sequestration analyses, we settled on N = 2-4 biological replicates given similar experiments in citations above, and each biological replicate contained pools of 50-100 individuals based on preliminary studies with standards.
Data exclusions	In the CAFE assay the vials in which all flies died early in combination with a lack of feeding were excluded from all analyses. These deaths were attributable to flies being unable to access the food. Further vials were excluded from LD50 and feeding rate measurements if there was a lack of feeding for one of the days (again the result of flies being unable to access the food) or if there were missing feeding data. These were pre-established exclusion criteria.
Replication	We performed all bioassays as well as enzyme assays in replicates. Results of all assays were reproducible. Please refer to Methods for numbers of replicates.
Randomization	We used random.org/sequences to randomize placement of tubes in racks for all bioassays, except for the feeding experiments with milkweed and the sequestration assays. For the milkweed feeding experiments, the initial experiment was done as a preliminary study without randomization and we did not anticipate it working because of the myriad compounds in the plants, which we thought would kill even the monarch genotype flies. The monarch flies, to our surprise, survived relatively well on media containing milkweed, but because milkweed was no longer available (we collected it on UC-Berkeley campus), except enough for one repeat of the experiment, we followed the first design (no randomization) to keep our design consistent. For the sequestration assays, puparia were transfered out of the vials to prevent freshly emerging flies from feeding on the media containing ouabain, after which flies were collected flies every two hours. For these reasons we did not randomize.
Blinding	We were blind to the allocation and outcome assessment during all bioassays, except for the feeding experiments with milkweed and the sequestration assays. For the milkweed experiments, the media is green and thus reveals to the experimenter which vial is in which treatment group. For the sequestration assays, the personnel who conducted the rearing were at a different university (Cornell University) than those who conducted the HPLC. In addition, we had to know which flies emerged from which vials to group them and freeze them. Given these procedures, we were not blind to the experimental treatment for these two experiments.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☐	☒ Unique biological materials
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials	All unique materials are available upon request. The knock-in fly lines will be available from the Bloomington Drosophila Stock Center or directly from N.K.W. (email to whiteman@berkeley.edu).

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	Sf9 cells in Grace's were originally purchased from Gibco™ , now owned by ThermoFisher, under catalog number B82501.
Authentication	From ThermoFisher's website (https://www.thermofisher.com/order/catalog/product/B82501?SID=srch-srp-B82501): "Each lot of Gibco® Sf9 cells is tested for cell growth and viability post-recovery from cryopreservation...and has been characterized by isozyme and karyotype analysis." In addition, the cells have been maintained at the University of Hamburg (Susanne Dobler) for the last 12 years and checked regularly for contamination by bacteria and mycoplasma.
Mycoplasma contamination	From ThermoFisher's website (https://www.thermofisher.com/order/catalog/product/B82501?SID=srch-srp-B82501): "In addition, the Master Seed Bank has been tested for contamination of bacteria, yeast, mycoplasma and virus..."
Commonly misidentified lines (See ICLAC register)	Cells are not listed in the database.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Only invertebrate animals were used in our experiments. We used eggs, larvae (1st-3rd instar), puparia and adults (females, aged 4-7 days) of the w1118 strain and the 8 knock-in lines we created in Drosophila melanogaster (Diptera, Drosophilidae) for all experiments. Please refer to the methods section for a description of the transgenics and genetics methods and validation. The Berkeley campus Committee on Laboratory & Environmental Biosafety has approved a Biological Use Authorization (#451) for the maintenance and disposal of the knock-in D. melanogaster lines generated by GenetiVision (Houston, TX), adhering to regulations of the National Institutes of Health in the laboratory of Prof. Noah Whiteman.
Wild animals	We did not use wild animals for the present study.
Field-collected samples	We did not use field-collected samples for the present study.