DAUGAVPILS UNIVERSITY
INSTITUTE OF LIFE SCIENCES AND TECHNOLOGY
DEPARTMENT OF BIODIVERSITY

SERGEJS POPOVS

Unpredictability as an Adaptive Behavioral Characteristic of Prey: the Case of Fruit Flies (*Drosophila melanogaster*)

Neprognozējamība kā upura adaptīvās uzvedības pazīme: augļu mušu (*Drosophila melanogaster*) piemērs

Doctoral Thesis
(thematically coherent set of scientific publications)
for obtaining the doctoral degree (Ph. D.) in Natural sciences
(Biology branch, Ecology sub-branch)

Promocijas darbs
(tematiski vienota zinātnisko publikāciju kopa)
zinātniskā doktora grāda (Ph. D.) Dabaszinātnēs iegūšanai
(Bioloģijas nozarē, Ekoloģijas apakšnozarē)

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DAUGAVPILS, 2025

The thesis was performed: in Latvia, at Daugavpils University, Institute of Life Sciences and Technology, Department of Biodiversity, Laboratory of Animal Ecology and Evolution in 2019 – 2025.

This thesis was supported by the European Social Fund project Nr. 8.2.2.0/20/I/003 and the Latvian Council of Science project Nr. lzp-2024/1-0437

Type of work: doctoral thesis (a set of publications) in Natural sciences, Biology branch, Ecology sub-branch.

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Commencement: Daugavpils Universitātes Bioloģijas zinātņu nozares promocijas padomes atklātā sēdē 2025. gada 28. aprīlī, plkst. 12:00.

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LIST OF ORIGINAL PAPERS

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I. Krams, I. A., Krama, T., Krams, R., Trakimas, G., **Popovs, S.**, Jõers, P., Munkevics, M., Elferts, D., Rantala, M. J., Makņa, J., & de Bivort, B. L. (2021). Serotoninergic Modulation of Phototactic Variability Underpins a Bet-Hedging Strategy in *Drosophila melanogaster*. *Frontiers in Behavioral Neuroscience*, 15. https://doi.org/10.3389/fnbeh.2021.659331

II. Krama, T., Munkevics, M., Krams, R., Grigorjeva, T., Trakimas, G., Jõers, P., **Popovs, S.**, Zants, K., Elferts, D., Rantala, M. J., Sledevskis, E., Contreras-Garduño, J., de Bivort, B. L., & Krams, I. A. (2023). Development under predation risk increases serotonin-signaling, variability of turning behavior and survival in adult fruit flies *Drosophila melanogaster*. *Frontiers in Behavioral Neuroscience*, 17. https://doi.org/10.3389/fnbeh.2023.1189301

III. Krama T., Bahhir D., Ots L., **Popovs S.**, Bartkevičs V., Pugajeva I., Krams R., Merivee E., Must A., Rantala M.J., Krams I., Jõers P. (2023). A diabetes-like biochemical and behavioral phenotype of *Drosophila* induced by predator stress. *Proceedings of the Royal Society B: Biological Sciences*, 290(2002): 20230442. DOI: 10.1098/rspb.2023.0442.

IV. **Popovs, S.**, Munkevics, M., Krama, T., Krams, R., Sledevskis, E., Trakimas, G., Zants, K., Grigorjeva, T., Mizers, V., Kolbjonoks, V., Jõers, P., & Krams, I. (2024). Explaining the survival of the sickest: Altered walking patterns are linked with improved adult survival in *Drosophila melanogaster* grown with predators during larval development. *Behaviour*, 161(2), 133–148. https://doi.org/10.1163/1568539X-bja10254

The author's contribution to the papers:

I PUBLIKĀCIJA

- apmēram 35 % no visa darba apjoma

II PUBLIKĀCIJA

- apmēram 30 % no visa darba apjoma

III PUBLIKĀCIJA

- apmēram 40 % no visa darba apjoma

IV PUBLIKĀCIJA

- apmēram 55 % no visa darba apjoma

CONTENTS

Approbation of research results at international scientific conferences:

1. Spanish Drosophila Meeting 2024, Sant Joan d'Alacant, Spain.
   Stenda referāts/poster "A diabetes-like biochemical and behavioral phenotype of *Drosophila* induced by predator stress".
   Krams I., Krama T., **Popovs S.**, Krams R, Jõers P.

2. Latvijas Universitātes 82. Starptautiskā zinātniskā konference, Rīga, Latvija.
   Mutisks ziņojums/talk "Altered walking patterns connection with improved survival in fruit flies (*Drosophila melanogaste*r) grown with predators during larval development".
   Kolbjonoks V., **Popovs S.**, Munkevics M., Krama T., Krams R., Sledevskis E., Trakimas G., Zants K., Grigorjeva T., Mizers V., Joers P., Indriķis Krams.

3. Latvijas Universitātes 82. Starptautiskā zinātniskā konference, Rīga, Latvija.
   Mutisks ziņojums/talk "Link between serotonin concentration changes and the lateral movements of fruit flies (*Drosophila melanogaster* Meigen, 1830)".
   Grigorjeva T., Kotova A., Maļutina V.V., Krama T., **Popovs S.**, Munkevics M., Zants K., Krams R., Joers P., Trakimas G., Krams I.

4. 27th European Drosophila Research Conference, Lyon, France.
   Stenda referāts/poster "Fear Me Not: Adaptive Metabolic and Behavioural Responses to Predator-Induced Stress in *Drosophila melanogaster*".
   Munkevics M., Krama T., **Popovs S.**, Krams R., Zants K., Grigorjeva T., Krams I.

5. Daugavpils Universitātes 65. starptautiskā zinātniskā konference, Daugavpils, Latvija.
   Mutisks ziņojums/talk "Biochemical and behavioral phenotype of fruit flies (*Drosophila melanogaster* Meigen, 1830) induced by predator stress".
   Popovs S., Krama T., Bahhir D., Ots L., Bartkevičs M., Pugajeva I., Grigorjeva T., Munkevics M., Zants K., Krams R., Merivee E., Must A., Rantala M.J., Krams I., Joers P.

6. Daugavpils Universitātes 65. starptautiskā zinātniskā konference, Daugavpils, Latvija.
   Mutisks ziņojums/talk "Effects of predator presence and 5-HT signaling on behavioral lateralization and survival in *Drosophila melanogaster*".
   Trakimas G., Krama T., Munkevics M., Krams R., Grigorjeva T., Joers P., **Popovs S.**, Zants K., Sledevskis E., de Bivort B.L., Krams I.

7. Daugavpils Universitātes 65. starptautiskā zinātniskā konference, Daugavpils, Latvija.
   Mutisks ziņojums/talk "What is personality?".
   Krams I., **Popovs S.**, Krams R., Zants K., Munkevics M., Trakimas G., Grigorjeva T., Sledevskis E., Krama T.

8. Daugavpils Universitātes 65. starptautiskā zinātniskā konference, Daugavpils, Latvija.
   Mutisks ziņojums/talk "The effect of serotonin concentration changes on the lateral movements of fruit flies (*Drosophila melanogaster* Meigen, 1830)".
   Grigorjeva T., Kotova A., Maļutina V.V., Krama T., **Popovs S.**, Munkevics M., Zants K., Krams R., Joers P., Krams I.

9. Latvijas Universitātes 81. Starptautiskā zinātniskā konference, Rīga, Latvija.
   Mutisks ziņojums/talk "Phototactic behavior of fruit flies (*Drosophila melanogaster* Meigen, 1830) study with the automated system".
   Grigorjeva T., Maļutina V.V., Kotova A., Krama T., Munkevics M., **Popovs S.**, Krams R., Krams I.

10. 11th International Conference on Biodiversity Research, Daugavpils, Latvija.
    Mutisks ziņojums/talk "Threat of predation alters *Drosophila melanogaster* movement patterns".
    Popovs S., Munkevics M., Trakimas G., Krama T., Krams R., Grigorjeva T., Krams I.

11. 11th International Conference on Biodiversity Research, Daugavpils, Latvija.
    Mutisks ziņojums/talk "Use of automated system to study the effect of serotonin concentration changes on the behaviour of fruit flies (*Drosophila melanogaster* Meigen, 1830)".
    Grigorjeva T., Maļutina V.V., Kotova A., Krama T., Munkevics M., **Popovs S.**, Krams R., Krams I.

12. The evolution of personality in animals and humans, Erice, Italy.
    Mutisks ziņojums/talk "High-throughput devices to study serotonin-dependent phototactic personality, behavioral lateralization, and explorative activity in *Drosophila melanogaster*".
    Krams I., Krama T., Krams R., **Popovs S.**

13. The evolution of personality in animals and humans, Erice, Italy.
    Stenda referāts/poster "Serotoninergic Modulation of the Variability in Phototactic Personality Underpins a Bet-Hedging Strategy in *Drosophila Melanogaster*".

Popovs S., Krams I., Krama T.

14. Daugavpils Universitātes 63. starptautiskā zinātniskā konference, Daugavpils, Latvija.
    Mutisks ziņojums/talk "Neurotransmitter Serotonin Acts as a Suppressor of Phototactic Choice Variability in *Drosophila Melanogaster*".
    Krams R., **Popovs S.**, Krams I., Krama T., Munkevics M., Elferts D., Makna J.

15. Latvijas Universitātes 79. Starptautiskā zinātniskā konference, Rīga, Latvija.
    Mutisks ziņojums/talk "Predator Stress Represses Systemic Glucose Use Through Serotonin-Dependent Effect".
    Krams I., Krams R., **Popovs S.**, Munkevics M., Krama T., Joers P.

16. Latvijas Universitātes 79. Starptautiskā zinātniskā konference, Rīga, Latvija.
    Mutisks ziņojums/talk "Spider Odors Induce Changes in Body Carbon and Nitrogen Concentrations in *Drosophila Melanogaster*".

    Munkevics M., Krams R., **Popovs S.**, Krams I.

1. INTRODUCTION

1.1. Mechanisms of phenotypic adaptation to fluctuating environments

Populations of organisms are constantly exposed to numerous variable biotic and abiotic factors. Organisms and their populations must respond to these ever-changing conditions by evolving various adaptations and survival strategies to process the information on environmental variability, react appropriately to increase fitness and avoid extinction. Three main mechanisms of phenotypic adaptation include adaptation through the continual natural selection on heritable variations (adaptive tracking), adaptive phenotypic plasticity, and bet-hedging. Charles Darwin suggested adaptive tracking as a survival mechanism more than a century ago (1859). Although this mechanism improves the viability of populations, it has some limitations regarding the individual level. Even though standing genetic variation of most traits can be successfully maintained by a balance between mutation and selection, acquiring novel traits is a relatively slow process when the rate of environmental change is high (Tufto, 2015).

Plasticity is another form of phenotypic adaptation. The phenotypic traits of organisms develop under the influence of genetic and environmental factors and their combinations. Ecological/physiological plasticity is considered a highly flexible mechanism. However, it also has some limitations in providing organisms with resistance to environmental and evolutionary factors. The main problem arises because plasticity is developed by the interaction between genes and the environment during development. In contrast, natural selection occurs in another environment sometime after development (Gavrilets & Scheiner, 1993). A third form of phenotypic adaptation is bet-hedging, which is a risk-spreading strategy to diversify phenotypes in a population randomly (Morawska et al., 2022). Although adaptive plasticity and bet-hedging are sometimes considered two competing survival and reproductive strategies under conditions of environmental heterogeneity (Draghi, 2023), the three mechanisms of phenotypic adaptation to fluctuating environments may co-occur.

1.2. Bet-hedging

The environment is rarely stable, requiring complex strategies for organisms to optimize chances of survival and reproductive success. When critical factors such as temperature,

precipitation, food resources, and predation threat exhibit high levels of variability, critical processes such as reproduction and offspring development are at risk. This defines a bet-hedging approach: rather than investing all resources in a single strategy that may fail when conditions change, organisms spread their risks to improve the overall chance of success over time (Chirgwin et al., 2015; Merilä & Hendry, 2014; Trakimas et al., 2019).

Bet-hedging is not limited to reproductive strategies; it can be applied to a variety of traits, including all kinds of behavior. For example, organisms can vary their behavior so that it appears unpredictable, thereby reducing the chances of negative impacts from unfavorable changes in the environment, such temperature- or predation-induced stress. Thus, behavioral flexibility can be considered as an adaptive response to environmental uncertainty (Olofsson et al., 2009; Siepielski et al., 2017).

1.3. Behavioral and metabolic consequences of stress

Predation is not reduced to the simple death of individual prey, but can induce long-lasting fear effects in large numbers of surviving individuals (Hossie et al., 2010; Lehmann et al., 2014). Fear stimulates fleeing and hiding behaviors, yet these changes in response indicate morphological and physiological changes occurring in the prey (Janssens & Stoks, 2014). Stress disorders following non-lethal encounters with predators have long-term and costly consequences (Zanette et al., 2019). Certainly, behavior and phenotype changes do follow metabolism, and we can relate here to studies of stress in humans. The underlying mechanisms of interaction are not clear, but it is known that chronic stress leads to the development of insulin resistance (Beaupere et al., 2021). For *Drosophila melanogaster*, this means switching the organism to other biochemical patterns, where a reduced ability to metabolize glucose (the main energy source for flies) forces a switch to increased fat consumption. Our studies have shown that this is due to a dramatic decrease in the activity of the central regulatory kinase Akt, an enzyme that plays an important role in glucose uptake (Huang et al., 2018).

The fact that glucose does not enter tissues capable of processing it may be an adaptation creating memories at the biochemical level and preparing for future stresses (Roozendaal, 2002). However, chronic suppression of glucose utilization due to stress, switches systemic metabolism

to fat usage (Tennessen et al., 2011). Loss of metabolic flexibility leads to decreased fitness due to reduced ATP production and poor resistance to nutritional deficiencies.

Metabolic disorders necessarily lead to changes in locomotor activity. Yet it is not only the intensity of movements to conserve energy that changes dramatically, but the whole pattern of walking and even standing in place. The measured movement has been replaced by sudden and short sprints, and the resting stops have changed their character. Unexpectedly, during rest periods, flies subjected to chronic predation stress expended a great amount of energy on senseless chaotic movements rather than preparing for the next dash. This reflects morbid changes in physiology characterized by a variety of coordinated symptoms such as anxiety, chaotic grooming behavior, and inability to concentrate (Hart, 1988).

It is traditionally believed that predators prefer easy prey: young, inexperienced or sick individuals (Genovart et al., 2010). In contrast, our studies have shown that despite the counterintuitive sickness-resembling behavior of *Drosophila* with high levels of stress, their survival rate is much higher than in control groups. This may be because the randomness of prey movements gives predators many false signals for immediate future actions (Bilecenoğlu, 2005; Card & Dickinson, 2008; Eifler & Eifler, 2014; Yager et al., 1990). It can also be hypothesized that some predators are selective in avoiding infected prey (Gutierrez et al., 2022; Hamilton & Zuk, 1982; G. A. Jones et al., 2005; Meyling & Pell, 2006).

1.4. Serotonin drives predictability

Bet-hedging might be a key strategy for adapting to environmental changes and predation threats, and we were able to establish that serotonin plays a particularly important role in this process. Observations have shown that serotonin regulates the predictability of light choices, a crucial behavior that allows flies to respond to unpredictable conditions. Evolutionary modeling suggests that this variability in behavior may not be random, but a deliberate survival strategy in which serotonin levels in the flies' brains adjust their ability to adapt (Kain et al., 2015).

This is extremely important in a variable climate, but potentially even more important when threatened by predation. Exposure to predators during critical developmental periods leads to changes in serotoninergic signaling, which increases behavioral variability and potentially

increases survival (Krama, et al., 2023; Maloney, 2021). This highlights how serotonin not only affects mood and general behavior, but is also critical for specific behavioral responses to avoid predators.

We hypothesize that serotonin plays a central role in the association between neurochemistry and metabolism. Serotonin has multiple biological functions in *Drosophila*: it regulates courtship behavior, affects spatial memory and olfactory learning, and influences phototactic and turning behavior (Anaka et al., 2008; Diegelmann et al., 2006; Kain et al., 2012; Zhang & Odenwald, 1995). It is also involved in several pathways that overlap with the roles of other neurotransmitters such as dopamine and octopamine (Kaplan et al., 2008). The roles of serotonin in neuronal networks are extremely diverse and can even be redundant, and its effects on metabolism have an unusual mechanism underlying them. Serotoninergic neurons are closely adjacent to insulin-producing neurons, and these two systems exist in constant interaction (Al-Zoairy et al., 2017). Increased serotonin levels have a favorable effect on metabolic balance, improving glucose homeostasis and increasing the amount of energy available. Also, through its effect on metabolism, serotonin can increase the chances of survival in the event of starvation.

Still, research on altered walking and survival patterns reveals an additional layer of adaptations that arise in response to predation. Reduced serotonin levels cause major changes in behavioral patterns. Decreased speed, extended stopping times, and unpredictability may reduce visibility to predators, improving chances of survival. Thus, serotonin serves as a regulator of a spectrum of adaptations from metabolic changes to behavioral strategies, highlighting its role as a critical mediator in adapting to changing environmental conditions and predation.

1.5. Aims and goals of the thesis

The main aim of the thesis is to investigate whether unpredictability can be considered an important adaptive strategy used by prey to avoid predation and what is the central mechanism controlling this characteristic.

D. melanogaster exhibits high inter-individual diversity in phototactic behavior. The first objective of this thesis was to investigate a possible link between choice unpredictability and serotonin **(I)** regulation. We took advantage of the fact that the fruit fly inhabits a wide variety of

regions around the world and tested how populations living in different climates make light or dark phototactic choices. The basic assumption was that flies living in stable climates would exhibit high predictability of behavior and high levels of serotonin. Consistent with our expectations, serotonin inhibitors increased the variability of choices in flies reared in stable equatorial climates.

Variable climatic conditions can be a tremendous stressor for organisms, but in the natural environment they also face other stressors throughout their lives, the main one being predation. A second thesis objective was to test whether predictability of behavior changes in flies exposed to non-lethal predation stress early in life **(II)**. We predicted that flies could exhibit high variability in the choice of movement direction and that this behavior could enhance survival when directly exposed to a predation threat. We also expected that these processes are controlled through serotonin signaling.

Chronic stress is known to affect the metabolism of animals. *Drosophila* serotoninergic neurons are located extremely close to insulin-producing neurons, so the next thesis objective was to find out how strongly both stress and serotonin deficiency might affect the metabolism of fruit flies **(III)**. We predicted that serotonin would prove to be a key element in the regulation of glucose metabolism, which would affect *Drosophila* fitness. We also hypothesized that predation would reduce resistance to hunger conditions and available energy for utilization.

High levels of individuality, unpredictability in directional choices, and reduced energy availability are supposed to cause changes in behavioral patterns. The next goal of the thesis was to understand exactly how the movements of chronically stressed *Drosophila* change and why this has a positive effect on survival **(IV)**. Based on data from previous experiments, we predicted a decrease in overall *Drosophila* activity. However, an unexpected finding was the data on resting behavior of flies - they expended considerable resources on meaningless chaotic movements. We hypothesized that in this way the prey gives the predator a large number of false signals about its future movements and thus becomes less predictable and conspicuous, which increases the chances of survival.

2. MATERIALS AND METHODS

2.1. *Drosophila* husbandry and food formulations (I, II, III, IV)

The study utilized wild strains Oregon-R-modENCODE (no. 25211) and w^{1118} of *D. melanogaster*, sourced from the Bloomington Drosophila Stock Center, Indiana, USA. The flies were maintained in incubators set to 23 ± 1 °C with a 12:12 h light-dark cycle to simulate natural conditions.

To facilitate breeding, 10 female and 5 male flies were placed together in vials measuring 24.5 × 95 mm. These vials were filled with 18 ml of a specially prepared food mixture, allowing the flies to copulate and lay eggs for a 24-hour period.

The food for the *Drosophila* consisted of a mixture combining 500 ml water, 20 g dextrose, 15 g sucrose, 10 g brewer's yeast, 35 g cornmeal, and 4.5 g agar. To prevent mold growth, 12.5 ml of a 10% Tegosept (methyl-p-hydroxybenzoate) solution was added. For certain experimental groups, the food was supplemented with metformin or 5-hydroxytryptophan (5-HTP, a precursor of 5-HT synthesis) at concentrations of 20 mM and 5%, respectively, after the mixture cooled to below 65 °C.

Vials containing *Drosophila* eggs were positioned within plastic jars (10 cm height × 12 cm diameter). In experimental setups involving predation, each jar housed a pirate otter-spider (*Pirata piraticus*), allowing it to freely enter the vials and predate on the *Drosophila* larvae.

For biochemical analyses, adult flies were collected within 5-7 hours post-imaginal eclosion and subsequently stored at -80 °C. Flies intended for behavioral assays were utilized within 2-3 days following eclosion.

The rate of feeding was assessed by providing flies with food supplemented with Blue FCF dye. Spectrophotometric analysis of homogenates was used to quantify dye uptake, indicating the amount of food consumed. Flies were initially recovered from CO_2 exposure in standard food bottles before being transferred to food with or without dye supplementation. The absorbance of

the supernatant from homogenized flies was measured at 650 nm, with values from flies on dye-free food serving for background subtraction.

2.2. Drug treatments (I)

We had three experimental groups per geographic location: flies (males and females) grown without any drugs, flies grown on food supplemented with 5-HTP and flies grown on food supplemented with α-methyl-tryptophan (αMW, a serotonin-synthesis inhibitor) (Dasari et al., 2007; Dierick & Greenspan, 2007; Hu et al., 2020; Majeed et al., 2016; Neckameyer, 2010; Ries et al., 2017). Drugs were dissolved in Formula 4–24 instant *Drosophila* media. For the drug-feeding, F0 flies laid eggs in drug-containing media. Upon eclosion, adult F1 flies were assayed on days 2–3. The drug stock solutions were vortex-mixed and added to food powder. The final concentration of 5-HTP was 50 mM and the final concentration of αMW was 20 mM (Huber, 2004; Kain et al., 2012).

2.3. Neurotransmitter treatments (II)

We had two main experimental groups of *D. melanogaster*: flies grown together with predators and flies grown with no predators; each of these two groups was further divided into three subgroups: flies raised on food supplemented with 5-HTP, flies grown on food supplemented with αMW, and flies grown without any drugs (Dasari et al., 2007; Dierick & Greenspan, 2007; Hu et al., 2020; Krams et al., 2021; Majeed et al., 2016; Neckameyer, 1996; Ries et al., 2017). The drug stock solutions were vortex-mixed and added to food powder. 5-HTP and αMW were dissolved in Cal Tech instant media (United States Biological, Salem, MA, USA). The final concentration of 5-HTP was 50 mM, and the final concentration of αMW was 20 mM (Kain et al., 2012; Krams et al., 2021). The flies were 5–7 days old at the moment of behavioral experiments. Dierick and Greenspan (2007), by using HPLC, showed that 5-HTP feeding significantly increases the brain 5-HT within 3 days of treatment, while αMW significantly decreases the amount of brain

5-HT during 4 days of treatment. Honegger et al (2020) confirmed similar effects (∼8× reduction of 5-HT with αMW treatment; ∼20× increase with 5-HTP) using ELISA assays.

2.4. Feeding experiments (III)

To measure the rate of feeding, food supplemented with blue dye (Blue FCF dye, Acros Organics A0373695, ThermoFisher Scientific) was fed to flies. The amount was quantified spectrophotometrically from homogenate. For each experiment, 140 flies from the control condition and 140 from the predator-stress condition were placed in two separate standard food bottles, and allowed to recover overnight from CO_2 exposure. On the next day, the flies were transferred without gas either to a new standard food or to food supplemented with 1% Blue FCF dye. After 1.5 h, 20 flies were collected and homogenized on ice by grinding in a mortar and pestle in 800 µl phosphate-buffered saline (PBS). Debris was pelleted at 10 000 gmax for 10 min at 4 °C, and 400 µl of each supernatant was transferred to 2 wells (200 µl each) of 96-well plates. Absorbance was measured at 650 nm, and values from lysates of flies kept on food without Blue FCF were used for background subtraction.

2.5. Survival under predation (II, III, IV)

To investigate the impact of predation and pharmacological manipulation on the survival of *Drosophila,* we structured our experimental design around various conditions involving predator exposure and drug supplementation. Our study comprised six experimental groups, assessing the effects of two spider species under three different drug conditions: (1) fruit fly males grown without any spider presence and without any drug treatments, (2) male flies raised without spiders, with food supplemented with 5-HTP, (3) male flies also grown without spider exposure, on food supplemented with αMW, (4) males raised in the presence of *P. apacheanus* spiders with no drug supplementation, (5) males grown with *P. apacheanus* spiders on 5-HTP supplemented food, and (6) males raised with *P. apacheanus* spiders with food supplemented with αMW.

For the survival assays, we used ten Plexiglas jars for each experimental group, each jar measuring 10 cm in height and 12 cm in diameter. Each jar housed ten fruit flies, summing up to 480 fruit flies across 48 jars, maintained for 12 hours during daylight time. To simulate predation stress, a single young *P. apacheanus* spider, around 6–7 months old, was placed in each jar along with a vial containing fruit fly food. Spiders had access to water but were deprived of food for approximately 10 hours before the start of the survival tests, ensuring each spider was used only once to maintain consistency.

In parallel, to assess the adaptive value of a diabetes-like phenotype under predatory conditions, we established 20 groups (10 experimental and 10 control), each comprising 10 male *Drosophila*. These groups were placed in plastic containers measuring 20 cm in width, 10 cm in depth, and 10 cm in height. Similar to the previous setup, each container housed a single predator - either a pirate otter-spider or a wolf spider, referred to in our experiment - and a vial with *Drosophila* food made of cornmeal, dextrose, sucrose, agar, and yeast medium. A layer of filter paper lined the bottom of each container, and the tops were covered with mash. Spiders were subjected to a 12-hour food deprivation period before the trials and provided water before and during the tests to standardize hunger levels. Surviving flies were counted after the 12-hour period to evaluate the effectiveness of the predation pressure and the potential adaptive value of the flies' physiological responses to it.

2.6. Starvation tolerance measurements (III)

In the chronic starvation tolerance test, flies were kept on 1% agar in tubes containing 10 individuals. Survival was monitored every 3 h. Death was determined as the last activity time point from the final recorded activity for each fly. In the acute starvation tolerance test, flies were starved on deionized water-soaked filter paper in tubes containing 10 individuals. The moisture content of the paper was controlled by injecting water with a syringe once a day.

2.7. Phototaxis equipment (I)

We studied the variability of phototaxis behavior in F1 flies. The FlyVac apparatus allowed us to measure the startled phototaxis behavior of many individual fruit flies simultaneously (Kain et al., 2012). The operational details of FlyVac are detailed elsewhere (Kain et al., 2012). In brief, FlyVac is an instrument for the rapid quantification of phototaxis behavior. Up to 32 individual fruit flies were loaded into separate phototaxis modules, each consisting of a phototactic T-maze in which the fly could choose between a light [an illuminated light-emitting diode (LED)] and dark stimulus (a non-illuminated LED). Both branches of the T-maze are equipped with an LED but only one LED is illuminated, at random, in each trial.

To begin a phototaxis session, individual flies are aspirated from its culture vial into the vertical start tube of the T-maze. After insertion, a fly climbs upward through the vertical tube of the T-maze under negative geotaxis until it reaches the choice point of the T-maze. Upon making a choice by entering one of the corridors of the T-maze, the fly is detected by an optical interrupter. This trigger recording the direction of the choice done with respect to the direction of the illuminated stimulus LED and opens a vacuum to pull the fly back into the start tube. In each trial, one LED out of two is lit at random. After completing 40 trials, the phototaxis module is deactivated and the flies are simply contained until removal. In the event that a fly does not complete 40 trials within several hours, that fly is removed from the module and further analyses. Before the trials, we have checked whether the FlyVac apparatus itself was not affecting behavior. We have performed a long series of assays with two LEDs on and with two LEDs off. In both cases, the resulting distributions are statistically indistinguishable from the random binomial distribution.

2.8. Turning behavior (II)

Since using variance as a phenotypic trait requires large sample sizes (Caballero et al., 2021), we used a high-throughput assay to monitor the behavior of individual flies placed into individual Y-mazes (Ayroles et al., 2015; Buchanan et al., 2015). We put flies into an array containing 95 individual Y-mazes consisting of three symmetrical arms (each 12 mm long)

fabricated from laser-cut acrylic. Maze arrays were illuminated from below with a grid of 100 white LEDs (5500K, Knema) below acrylic diffusers. Maze arrays were imaged with 2MP digital cameras (Point Gray), and the X-Y positions of each fly's centroid were automatically tracked and recorded with software custom written in LabView (National Instruments, USA) (Buchanan et al., 2015). We recorded the turning behavior of 3–6-day old flies, the standard age for measuring this behavior, for 2 h. Data from the small portion of individuals making fewer than 30 turns were discarded. Each fly was used only once.

To quantify turning predictability (the variability in turning bias across individuals), we computed the MAD, the median of the absolute deviation from each observation's median (Buchanan et al., 2015), a metric of variability that is robust to outliers. We estimated MAD for each experimental group.

2.9. Behavioral assays (III)

We used sterile Petri dishes molded from clear polystyrene (60 × 15 mm; Flystuff, El Cajon, USA) as novel arenas to record individual flies' locomotor activity. Only one fly was aspirated into the arena for each test. The locomotor activity of six flies was recorded with the resolution of 1920 × 1080 pixels at 5 frames per second simultaneously by a video-tracking system using the Logitech HD Pro Webcam C920 (Logitech Inc., Newark, CA, USA), fixed at a height of 25 cm above the arenas, and the software Debut Video Capture (NCH Software, Greenwood Village, CA, USA). To shorten the experiment duration, two identical video-tracking systems were prepared, which allowed tracking of 72 flies simultaneously. The video-tracking course was 15 min. We calculated the flies' average speed for each minute. The arenas were illuminated by reflected, diffused light from above by four MR 16 LED lamps (12 V, 6 W, 400 lm, 3000 K) located 0.9 m above the arenas. Illumination at the level of the arenas (3000 lux) was measured by a TES-1335 Digital Light Meter (TES Electrical Electronic Corporation, Taipei, Taiwan). All video recordings were made in the laboratory at between 21 and 22 °C, and 35–40% relative humidity. Distance moved (start speed > 0.20 mm s^{-1}; stop speed < 0.20 mm s^{-1}) with the temporal bin width of 1 min as the most important locomotor activity parameter was extracted offline from the recorded video files using EthoVision XT Version 11 software (Noldus Information

Technology, Wageningen, The Netherlands). The distances moved were used to calculate the speed, representing the integral values of distances and time.

2.10. Mobility parameters of fruit fly walks (IV)

A plate with Y-shaped mazes was made for this study (Buchanan et al., 2015; Krama, et al., 2023). Each plate consisted of two layers: the first layer was made of solid transparent plastic; the second layer, with 60 mazes carved into it, consisted of black matte plastic to reduce light reflections. Each maze consisted of three sleeves equally spaced 120 degrees apart, each 3 mm wide and 12 mm long. Each arm ended in a circular turn with a diameter of 5 mm. Each maze was individually closed by a triangle of thin glass projecting above the plate surface. The glass was coated with Sigmacote (Sigma-Aldrich, St. Louis, MO, USA) to make it slippery and prevent the flies from turning upside down and walking on the ceiling. The height of each maze was 2 mm. In this way, all the flies had enough space to move freely but could not flip over and reduce their speed because of insufficient adhesion to the surface of the glass. The plate with mazes was illuminated from below through a thick matte plastic to create a contrasting surface for further recording of the movements of each fly. The recording was done in darkness to avoid the light reflections on the glass, which would have obstructed an accurate analysis of the movements. A Basler Ace camera with a 1/1.8″ sensor (Basler, Ahrensburg, Germany) and Kowa F1.6/4.4-11 mm optics (Kowa Optimed Germany GmbH, Duesseldorf, Germany) was mounted above the plate. Custom settings were chosen to ensure the highest accuracy and lowest distortion.

Each fly was gently placed in one maze, using a short carbon dioxide anesthesia. All flies were given at least 25 min to adapt after awakening. This was followed by two hours of continuous recording of the walking behavior of fruit flies. Each fly only participated in one trial. The video files were subsequently uploaded to Noldus EthoVision XT v.15.0 (Noldus Information Technology, Wageningen, The Netherlands) and analyzed using the following parameters: Distance Moved (mm), High Acceleration State frequency (see below), Maximum Acceleration (mm/s^{-2}) and Motion Without Movement frequency. These are the most important parameters by which insect movement patterns can be characterized (Nilsson & Renshaw, 2004; Russig et al., 2003; Winberg et al., 1993). For each metric, data was obtained as a mean value per individual fly.

The Acceleration metrics were used to mark bursts of rapid movement. The High Acceleration State was observed when the average acceleration of the object exceeded the 2.5 mm/s^2 threshold. The threshold value was adjusted by using the EthoVision XT Integrated Visualization tool. We used averaging interval of 2 to remove the effect of random changes in velocity between consecutive samples that would result in false transitions to High Acceleration State. The optimal state duration threshold was defined as 0.5 s and was found using the Integrated Visualization plot (i.e., we did not consider accelerations with a duration of less than half a second). It was used to filter out false readings from the body-point jitter that can be introduced by camera vibrations or minor body motions. The frequency of the High Acceleration state is presented as the median of all values for each group. Readings were recorded for the entire duration of the experiment.

Maximum Acceleration is presented as the median of all values for each group. Before calculating the acceleration, we ensured that the proportion of lost samples was less than 1%.

Distance Moved was determined within 2 h periods. We used a sample rate of 6 data points (according to Noldus). Higher values can lead to false readings and overestimation of the covered distance. On the other hand, small movements of the animal's central point may be missed due to lower values (Pham et al., 2009).

2.11. Fruit fly motions without movements (IV)

Motion Without Movement ("Mobility" in the Noldus software) describes the degree to which an object's body moves without regard to the spatial displacement of the central point. This implies that measurements are taken only when there is no movement of the animal's central point in the horizontal plane. *Drosophila* flies often perform "stomping in place" type behaviors. To describe this motion, calculations do not require x and y coordinates but instead, use the change in the position of individual pixels. This is an important parameter to estimate the degree of an animal's motion regardless of its locomotion along the x and y axes. A classic example of this parameter is animal grooming: although the animal's limbs and body are busy, the animal remains in one place.

We estimated the frequency of Motions Without Movements ("Highly Mobile" according to Noldus) using a threshold of 50% change in the pixel area of the detected subject. We used the default Averaging Interval set to 1 data point, which means that the measures are not smoothed before determining values.

One limitation of Motion Without Movement is that it directly depends on the number of pixels that compose the object under examination and, consequently, on the camera resolution. *Drosophila* is a small object consisting of approx. 100 ± 20 pixels, so we set an extremely high Immobility threshold of 50%. This means that the animal's motion was counted only if 50% of the pixels changed their position. In this way, we excluded the probability of recording false readings. To avoid false readings, we do not report the Immobility metric here.

2.12. Western analyses (III)

Batches of 30 flies were homogenized with a pestle on ice in 300 µl of western lysis buffer (PBS with 1.5% Triton X-100) supplemented with protease and phosphatase inhibitor cocktails (Roche Complete Mini no. 11836170001 and PhosSTOP no. 04906845001) following the manufacturer's protocols. Lysates were incubated on ice for 15 min and then centrifuged at 13 000gmax for 15 min at 4 °C to pellet debris. Supernatant protein concentrations were measured using the Bradford assay (Thermo no. 1856209), and 70 µg aliquots were loaded onto precast Bio-Rad Criterion AnyKD gradient gels. Gels were run in ProSieve EX running buffer (Lonza). Proteins were transferred to Amersham Protran nitrocellulose membrane (no. 10600020) in ProSieve EX transfer buffer (Lonza) at 35 V for 50 min in a BioRad Criterion Transfer chamber. Membranes were incubated in 5% BSA in 1× TBS/0.05% Tween for 1 h for blocking, after which they were incubated overnight at 4 °C in the same buffer with primary antibodies. Antibodies and dilutions used were: Akt 1: 5000 (Cell Signaling no. 9272), phospho-Akt 1: 5000 (Cell Signaling no. 4054), ACC 1: 5000 (Cell Signaling no. 3676), HRP-conjugated anti-rabbit 1: 10 000 (PI-1000-1).

After washing membranes three times for 15 min with 1× TBS/0.05% Tween, they were incubated with anti-rabbit secondary antibody conjugated with horseradish for 1 h at ambient room

temperature. After an additional three rounds of washing as before, results were visualized with the BioRad ChemiDoc XR detection system. For quantitation purposes, samples from control and predator-reared flies were run on the same gel with four individual biological replicates per group. When protein amount per lane was used for normalization, membranes were stained with Ponceau S solution (0.1% Ponceau S in 5% acetic acid), rinsed briefly with water, and documented using the BioRad ChemiDoc XR system. The signal was quantified, and the data were analyzed with ImageQuant software.

2.13. Metabolite analyses (III)

For carbohydrate measurements, 10 flies were homogenized in 400 µl of PBS and incubated for 5 min at 70 °C. A total of 40 µl of lysate was transferred to four separate Eppendorf tubes with additions of 1 U of amyloglucosidase from *Aspergillus niger* (Sigma, total glucose measurement), 2× PBS (free glucose and background measurement) and 5 mU of porcine kidney trehalase (Sigma T8778, trehalose measurement). All reactions were incubated for 2 h at 37 °C, after which they were briefly centrifuged, and 30 µl of supernatant was transferred to 96-well microtiter plates. One hundred microliters of Glucose Assay Reagent (Sigma G3293) were added to all reactions except for one PBS-treated lysate mixed with 100 µl of PBS to measure the background signal. Reactions were incubated at 37 °C for 30 min, after which absorption was measured at 340 nm. Free glucose, glycogen and trehalose were calculated by subtracting relevant backgrounds from measured values. A glucose standard curve was generated using 1 to 20 µg of glucose (per well). The results were normalized against protein amount measured with Bradford assay (Thermo no. 1856209).

For triglyceride measurements, 10 flies were homogenized in 800 µl of PBS with 0.1% Tween 20 and incubated for 5 min at 70 °C. Twenty microliters of each lysate were transferred to three Eppendorf tubes with additions of 20 µl of Triglyceride Reagent (Sigma T2449, total glycerol measurement) and 2 × 20 µl of PBS (free glycerol and background measurement). All reactions were incubated for 30 min at 37 °C, then briefly centrifuged, and 30 µl of supernatant was transferred to 96-well microtiter plates. One hundred microliters of Free Glycerol Reagent (Sigma F6428) were added to all reactions except for one PBS-treated lysate mixed with 100 µl of PBS to

measure the background. Reactions were incubated at 37 °C for 5 min, after which absorption was measured at 540 nm. Triglycerides were calculated by subtracting free glycerol from total glycerol measurement. A glycerol standard curve was calculated using 0.5 to 3 µg of glycerol (per well). The results were normalized against protein amount measured with Bradford assay (Thermo no. 1856209).

ATP concentration was measured using the ATP Determination kit (ThermoFisher Scientific). Thirty flies were homogenized in ATP isolation buffer (6 M guanidine-HCl, 4 mM EDTA, 100 mM Tris/Cl pH 7.8) and snap-frozen in liquid nitrogen, followed by boiling for 5 min. Debris was pelleted by centrifugation at 10 000gmax for 10 min at 4 °C. Five microliters of a 12.5-fold diluted supernatant was added to 100 µl of ATP Reaction Mix (Thermo Fisher; formulated according to the manufacturer's recommendations), and values were recorded using a Tecan luminometer with Greiner polypropylene plates (no. 655207). The results were normalized against protein amount measured with Bradford assay (Thermo no. 1856209).

Pyruvate was measured using BioVision kit no. K709 according to the modified protocol provided by the manufacturer. For pyruvate measurements, 20 flies were homogenized in 200 µl Pyruvate Assay Buffer on ice and then centrifuged at 10 000gmax for 10 min at 4°C. Fifteen microliters of supernatant were mixed with 35 µl of Pyruvate Assay Buffer in a well of the 96-well microtiter plate. Fifty microliters of reaction mix (formulated according to the manufacturer's guidelines) were added to each well containing supernatant and incubated for 30 min at room temperature, after which absorption was measured at 570 nm. Parallel background reactions were performed by mixing supernatant with background mix, formulated according to the manufacturer's guidelines. The results were normalized against protein amount measured with Bradford assay (Thermo no. 1856209).

2.14. Respiration exchange ratio measurements (III)

Respiration exchange ratio (RER) was calculated as the ratio of CO_2 produced and O_2 used by flies. O_2 consumption in individual flies was measured by coulometric respirometry in a continuous O_2-compensating system at constant temperature and humidity (23 °C and 55% relative

humidity). Flies were placed into measuring chambers, and measurements were begun when the flies stopped moving and the minimum value of gas exchange was reached. CO_2 levels were determined using a LI-700 differential CO_2/H_2O analyzer (LiCor, Lincoln, Nebraska, USA).

2.15. Data analysis and statistical methods (I, II, III, IV)

We employed a Poisson generalized linear model (GLM) with a log link function, considering positive light-choice count as the dependent variable. This model incorporated geographic location (Finland, Kenya), treatment groups (control, 5-HTP, αMW), and sex as predictors, with an interaction term for sex and treatment groups. Upon discovering that sex did not significantly influence the results, it was removed from the model, leading to a refined GLM that focused on the interaction between geographic location and treatment groups. The variability of phototactic choices was assessed using the variability beyond expectation (VBE) metric, a measure derived from the mean absolute deviation of data from their median, adjusted for expected sampling error, with standard errors for VBE calculated through bootstrap resampling of individual flies (5,000 replicates).

Further statistical analyses included permutation tests for comparing behavioral mean absolute deviations (MADs) across groups, with *P*-values determined by shuffling data tables and comparing randomized groups against original data. The impact of developmental conditions and drug supplementation on adult fly survival under predation was evaluated using two-way ANOVA, followed by Tukey's honest significance test for multiple comparisons, alongside the Kruskal–Wallis and Mann–Whitney U tests to compare behavioral traits such as turn bias and locomotory speed.

All analyses were performed in the R environment (version 4.1.0), ensuring rigorous statistical evaluation and adjustment for multiple comparisons using the Benjamini–Hochberg procedure (Benjamini & Hochberg, 1995). Biological replicates ranged from four to ten, with individual data points indicated on diagrams, and statistical significance set at $P < 0.05$.

For locomotor activity and survival analyses, data were analyzed using both generalized linear models and non-linear regression models, fitting data to specific distributions based on the nature of the data collected, including gamma and quasi-Poisson distributions for traits such as maximum acceleration and high acceleration state frequency. Survival analysis under predation employed a binomial distribution model, with all models considering statistical significance at $P < 0.05$.

GraphPad Prism software was utilized for graphical representation and further statistical calculations, including two-tailed Student's *t*-tests for comparing control and predator-reared populations, and Mantel–Cox tests for survival analyses. The application of the Lowess track smoothing method facilitated the nuanced interpretation of motion data.

3. RESULTS

3.1 Light-Choice Probability (I)

Female and male flies did not differ significantly in their LCP (fraction of choices toward the illuminated LED) either in Finland or Kenya (Poisson GLM with log link, Wald $Chisq_1 = 0.66$, $P > 0.4$) and, therefore, we pooled sexes in the further analyses of LCP. We found that geographic location was a significant predictor of light-choice probability (Poisson GLM with log link, Wald $Chisq_1 = 290.8$, $P < 0.0001$). Flies were photopositive both in Kenya and Finland, choosing the light 80% and 68% of the time (Table 1; Fig. 1), respectively. Kenyan flies were found to be significantly more photopositive than Finnish flies ($P < 0.001$; Table 1; Fig. 1). While the main effect of treatment group on light-choice was not significant (Poisson GLM with log link, Wald $Chisq_2 = 0.449$, $P = 0.8$), however, there was significant interaction between geographic location and treatment group (Poisson GLM with log link, Wald $Chisq_2 = 40.93$, $P < 0.0001$). Feeding Kenyan flies, αMW increased their LCP significantly while feeding 5-hydroxytryptophan, a serotonin precursor reduced LCP (Table 1; Fig. 1). In contrast, feeding Finnish flies αMW reduced LCP, while 5-HTP increased their LCP. However, LCP of Finnish flies fed 5-HTP was still lower than the LCP of Kenyan flies (Table 1; Fig. 1).

Geographic location	Treatment	*n*	Light choice probability (LCP) ± SE	Variability beyond expectation (VBE) ± SE
Finland	5-HTP	286	0.695 ± 0.00665	0.5278 ± 0.06943
Finland	Control	255	0.6798 ± 0.00779	0.7225 ± 0.0703
Finland	αMW	201	0.6454 ± 0.00977	0.8394 ± 0.07307
Kenya	5-HTP	175	0.7673 ± 0.00745	0.5458 ± 0.085
Kenya	Control	256	0.7964 ± 0.00564	0.3839 ± 0.07685
Kenya	αMW	197	0.8379 ± 0.00663	0.6392 ± 0.07468

Table 1. Descriptive statistics of light-choice probability and variability beyond expectation; ± SE were based on bootstrap resampling (5,000 replicates).

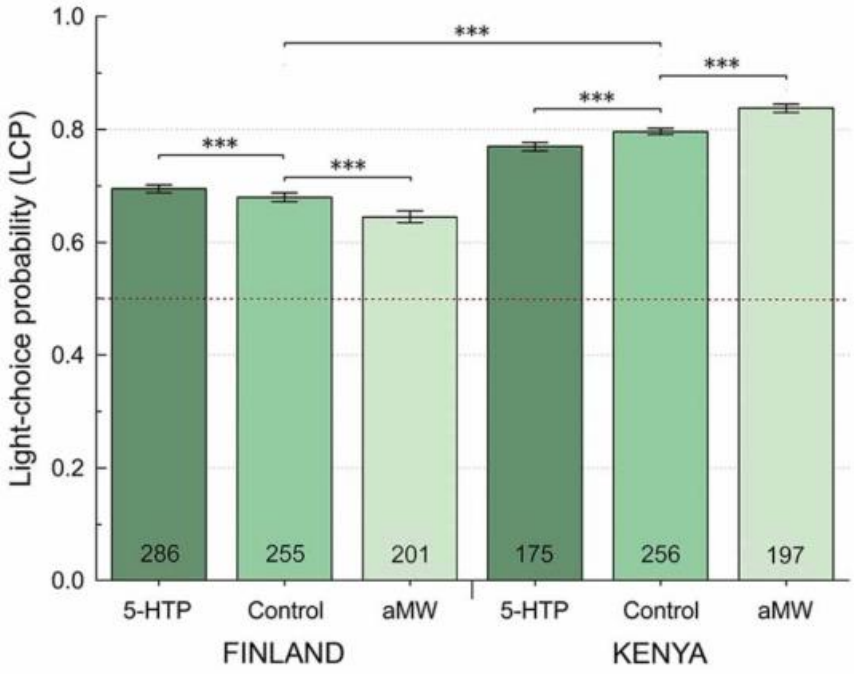

Figure 1. Light-choice probability by site and pharmacological treatment. Error bars are ± 1 SE, calculated by bootstrap resampling; ****P* < 0.001.

3.2 Variability Beyond Expectation (I, II)

Female and male flies had similar among-individual phototactic variability, as measured by VBE, in both Finland and Kenya (all bootstrapped distributions, $P > 0.05$) and sexes were pooled in the further analyses of VBE. Finnish fruit flies had significantly higher VBE than flies in Kenya ($P < 0.001$; Table 1; Fig. 2). Feeding αMW did not affect the VBE of Finnish flies, whereas adding 5-HTP to their food significantly suppressed VBE ($P = 0.023$). In Kenyan flies, feeding αMW significantly increased VBE, while 5-HTP did not affect their VBE (Table 1; Fig. 2). Importantly, feeding 5-HTP made VBE of Finnish flies similar to VBE of Kenyan flies (Table 1; Fig. 2).

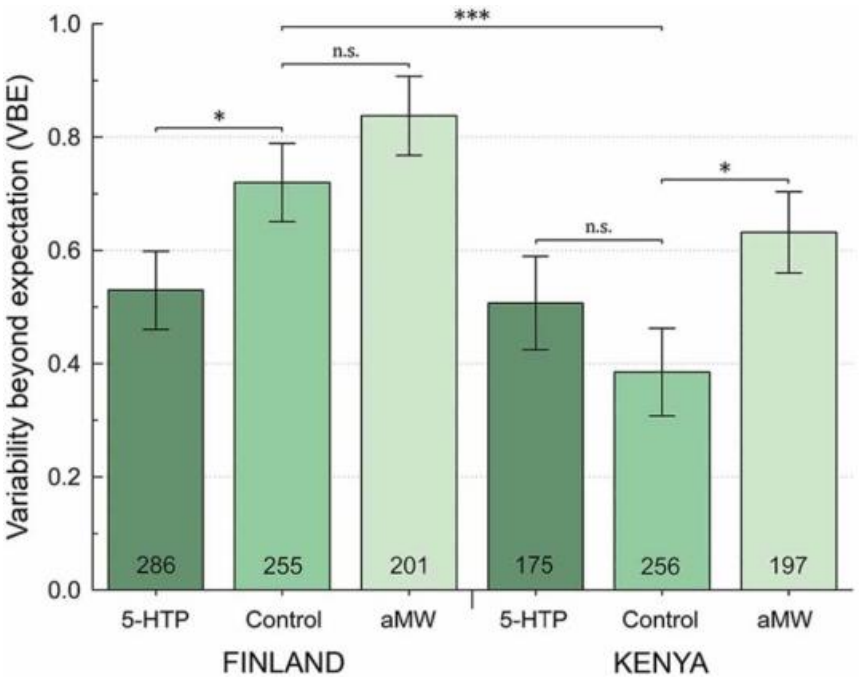

Figure 2. Behavioral variability beyond statistical expectation by site and pharmacological treatment. On a log2 scale, VBE = 0 indicates no excess variability, and VBE = 1 indicates twice as much variability as would be expected by chance alone. Error bars are ±1 SE, calculated by bootstrap resampling; n.s., $P > 0.05$, *$P < 0.05$, ***$P < 0.001$ represent one-tailed significance.

Turn bias variability of male fruit flies grown with spiders (MAD = 0.11, n = 153 flies) was significantly higher than that of control flies (MAD = 0.08, n = 143) grown in a predator-free environment (Permutation test: $P = 0.006$; Fig. 4). Feeding 5-HT to flies reared with spiders (MAD = 0.12, n = 116) did not increase the turning variability ($P = 0.34$) while feeding these flies αMW (MAD = 0.10, n = 140) significantly decreased turn bias variability ($P = 0.021$; Fig. 3). Feeding 5-HTP ($P = 0.33$) and αMW ($P = 0.12$) did not affect the variability of turning behavior of control fruit flies (Fig. 4).

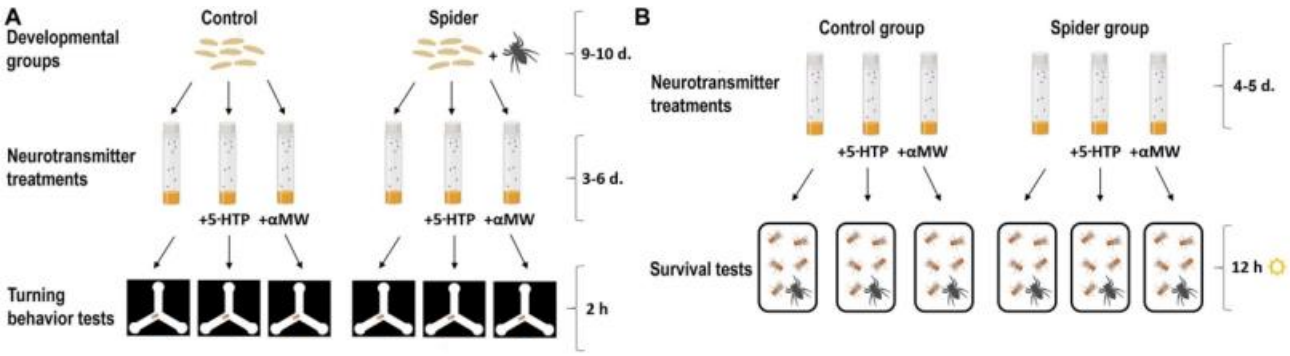

Figure 3. Schematic of the turning assay (A) and the survival experiment (B)

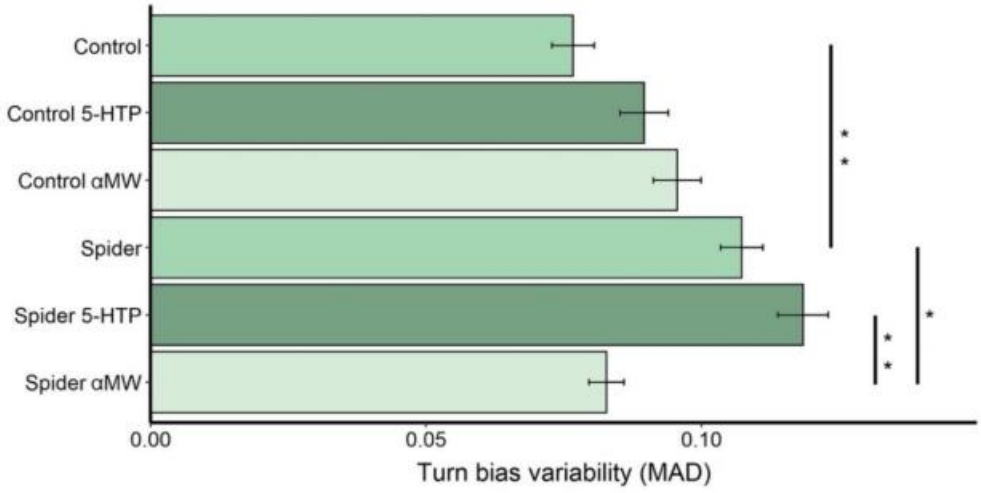

Figure 4. Turn bias variability (MAD) of fruit flies reared with and without spiders receiving different drug treatments. Error bars are ± SE estimated by bootstrap resampling. Asterisks indicate significant differences according to permutation tests: *0.05 > *P* > 0.01; **0.01 > *P* > 0.001.

3.3 Handedness and the number of turns in the y-maze (II)

The proportion of the right turns (turn bias) did not differ among the groups of flies (Kruskal–Wallis: $\chi^2 = 6.41$, $P = 0.268$; Fig. 5). Proportion of right turns by each group was also

not significantly different from 0.5 (Wilcoxon tests: all *P*s > 0.05; Fig. 5), i.e., an equal number of right and left turns in each group.

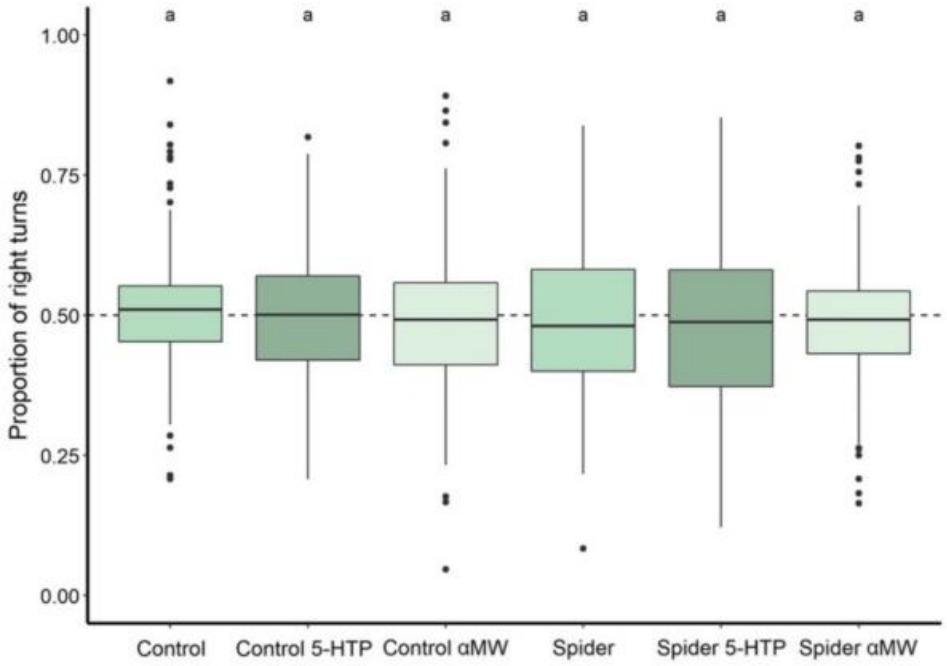

Figure 5. Turn bias of fruit flies reared with and without spiders and receiving different drug treatments during development. Experimental groups that are not statistically significantly different (Wilcoxon tests, $P > 0.05$) are indicated by the same letter at the top of the figure.

Flies reared with spiders made significantly fewer turns per unit time (2.6 ± SD 1.3 turns/min) in the Y-maze compared to control flies (3.4 ± 1.5 turns/minute) (Mann–Whitney test: $P = 0.0001$; Fig. 6). Feeding 5-HTP to flies reared with spiders significantly increased the turn rate (3.4 ± 1.4 turns/min) ($P < 0.0001$), whereas feeding them αMW had no significant effect (2.7 ± 1.3 turns/min) ($P = 0.50$; Fig. 6). Feeding αMW to control flies significantly decreased the turn rate (2.6 ± 1.51 turns/min) ($P = 0.0003$), whereas feeding them 5-HTP had no significant effect (3.5 ± 1.71 turns/min) ($P = 0.94$; Fig. 6).

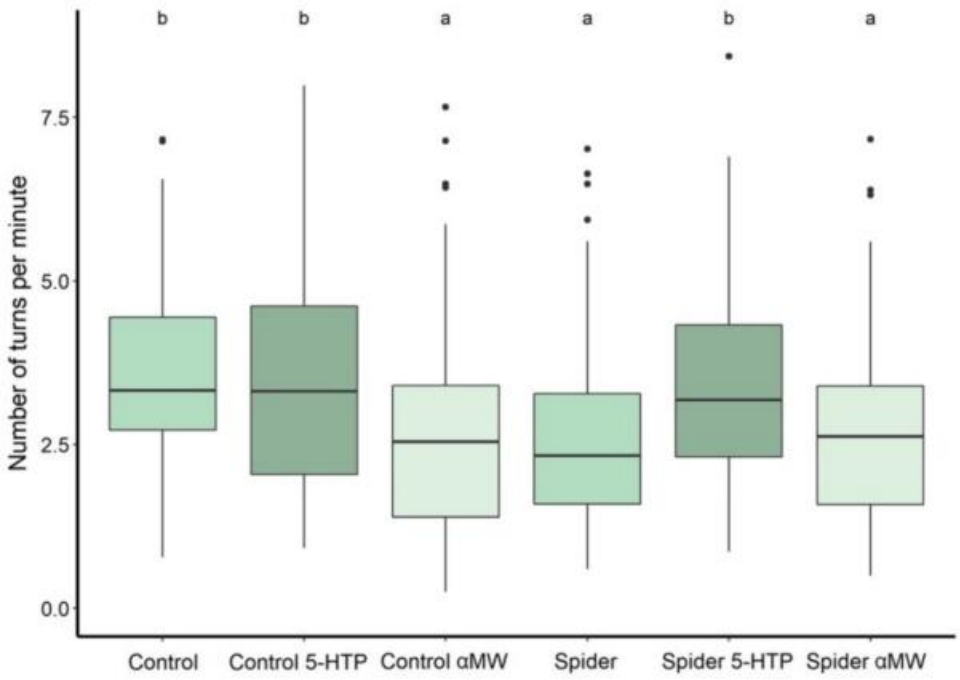

Figure 6. Turn rate (turns/minute) in the Y-maze of flies reared with and without spiders receiving different drug treatments. The flies reared with predators were previously exposed to predation during the larval stage, while in the control group, the flies were raised without jumping spiders. Thick lines indicate the median, boxes show the Q1 and Q3 quartiles, and whiskers represent the upper and lower quartile, excluding outliers. Black dots represent outliers (data points more than 1.5 times interquartile range away from Q1 and Q3). Experimental groups that are not statistically significantly different (Mann–Whitney tests, $P > 0.05$) are indicated by the same letter at the top of the figure.

3.4 Predator stress induces a catabolic shift towards lipid oxidation (III)

Both carbohydrates and lipids, as key biochemical energy storage molecules, were measured in *Drosophila* Oregon strain flies reared with and without predatory spiders. While free glucose, its disaccharide trehalose, and polymeric form glycogen ($n = 8$) remained stable regardless of predator stress, triglycerides decreased, and free glycerol increased compared with controls ($n = 10$, Fig. 7a, b). This indicates increased utilization of lipids since lipolysis of triglycerides would provide free fatty acids for catabolism and simultaneously increase free glycerol concentration.

Such specific loss of fat stores without any change in carbohydrate concentrations strongly indicates a shift in catabolism rather than inducing an overall starvation phenotype. Indeed, the RER (n = 20) of 0.76 in spider-reared flies supported this interpretation (Fig. 7c), indicating a firm reliance on a fatty acid breakdown in fueling systemic ATP production.

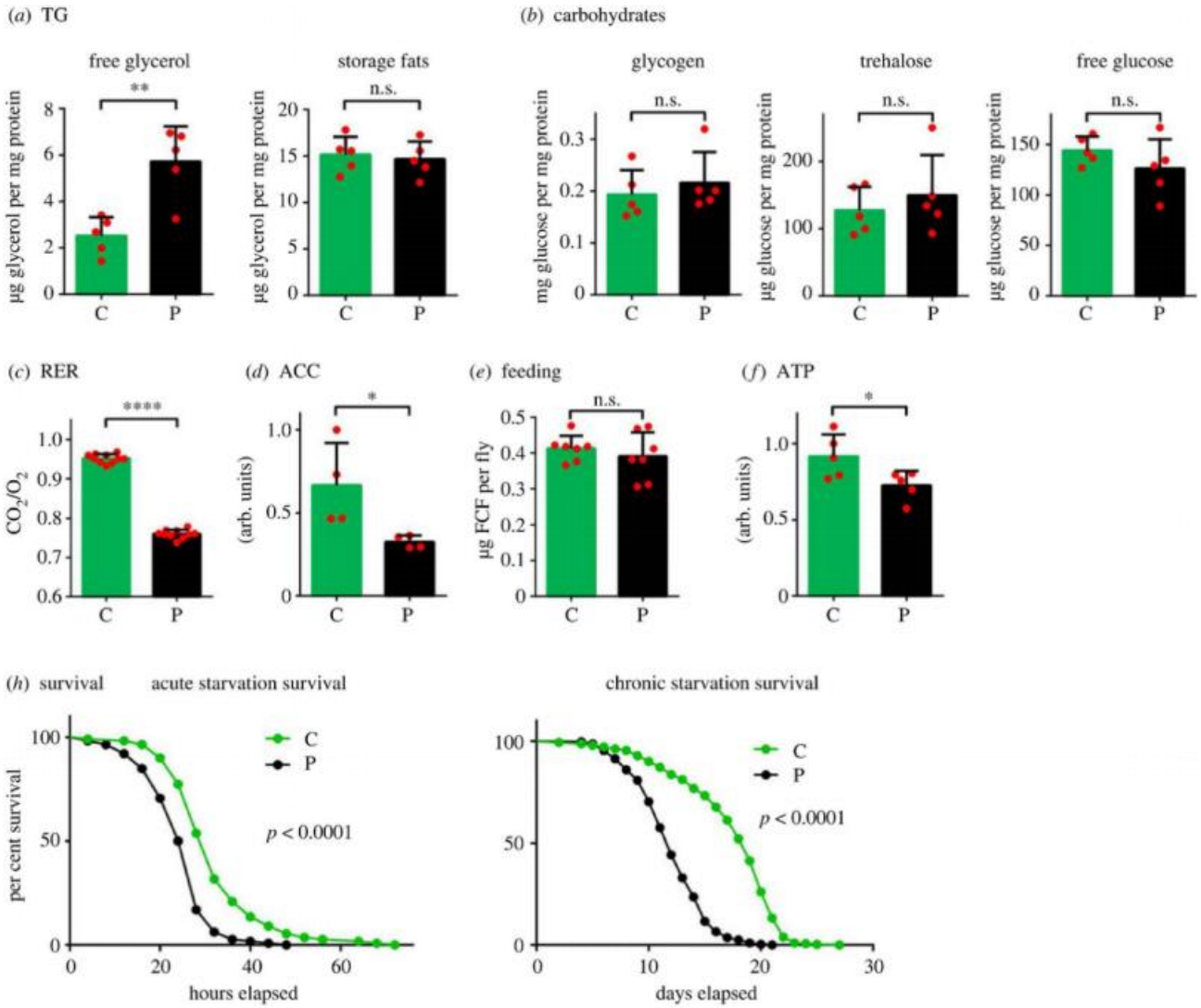

Figure 7. Effects of predator stress on the metabolism, locomotor activity, and survival in flies reared with spiders (predators) or without spiders (control). (a) Levels of free glycerol and storage fats. Relative but not absolute values of control flies have been published before (Krams et al., 2020). TG, triglyceride. (b) Levels of carbohydrates: glycogen, trehalose, and free glucose. (c) Respiration exchange ratio. (d) Amount of acetyl-CoA carboxylase (ACC) quantified against Ponceau S-stained total protein. (e) Uptake of food containing 1% Blue FCF dye. (f) ATP

concentration. (h) Survival curves of flies kept on agar food (acute starvation, log-rank test $P < 0.0001$) and on 1% sucrose food (chronic starvation, log-rank test $P < 0.0001$). In all cases: *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, n.s.—not significant. C—control, P—predator-reared.

3.5 Predator stress reduces overall energy levels (III)

Even if catabolism is re-oriented towards fatty acid oxidation, carbohydrates can contribute to this through de novo lipid synthesis. However, the levels of the rate-limiting ACC controlling this process were decreased in flies experiencing predator stress ($n = 8$, Fig. 7d). Increased feeding intensity ($n = 14$), a typical response to resource scarcity in *Drosophila*, was not found (Fig. 7e). Complete reliance on only one type of catabolic fuel source caused a 20% decrease in steady-state ATP levels ($n = 10$, Fig. 7f). Not compensating for diminished ATP production by increasing food uptake or lipid synthesis must come at the cost of lower metabolism. Spider-reared flies were indeed observed to have lower speed than controls in walking/climbing assays ($n = 24$ and 16, Fig. 9g). Similarly, these flies were less resistant to both acute ($n = 220$) and chronic ($n = 274$ and 275) starvation, exhibiting shorter survival in conditions of limited food resources (Fig. 7h).

3.6 Glucose uptake is inhibited (III)

The activity of Akt, a central regulator of the conserved glucose uptake mechanism, is dependent on the phosphorylation state of threonine at its kinase domain and serine residue in its hydrophobic motif (at position 505 in *Drosophila* Akt), which was found to be significantly decreased in spider-reared flies ($n = 8$, Fig. 8a). This indicates reduced glucose transport, depriving glycolysis of its substrate and decreasing its end-product pyruvate ($n = 4$, Fig. 8b). Administering metformin, an anti-diabetic drug that facilitates glucose uptake in both humans and flies (Bahhir et al., 2019; Niccoli et al., 2016), restored the normal balance in the flies' carbohydrate/lipid usage ($n = 10$) and increased their RER ($n = 20$) to normal value (Fig. 8c).

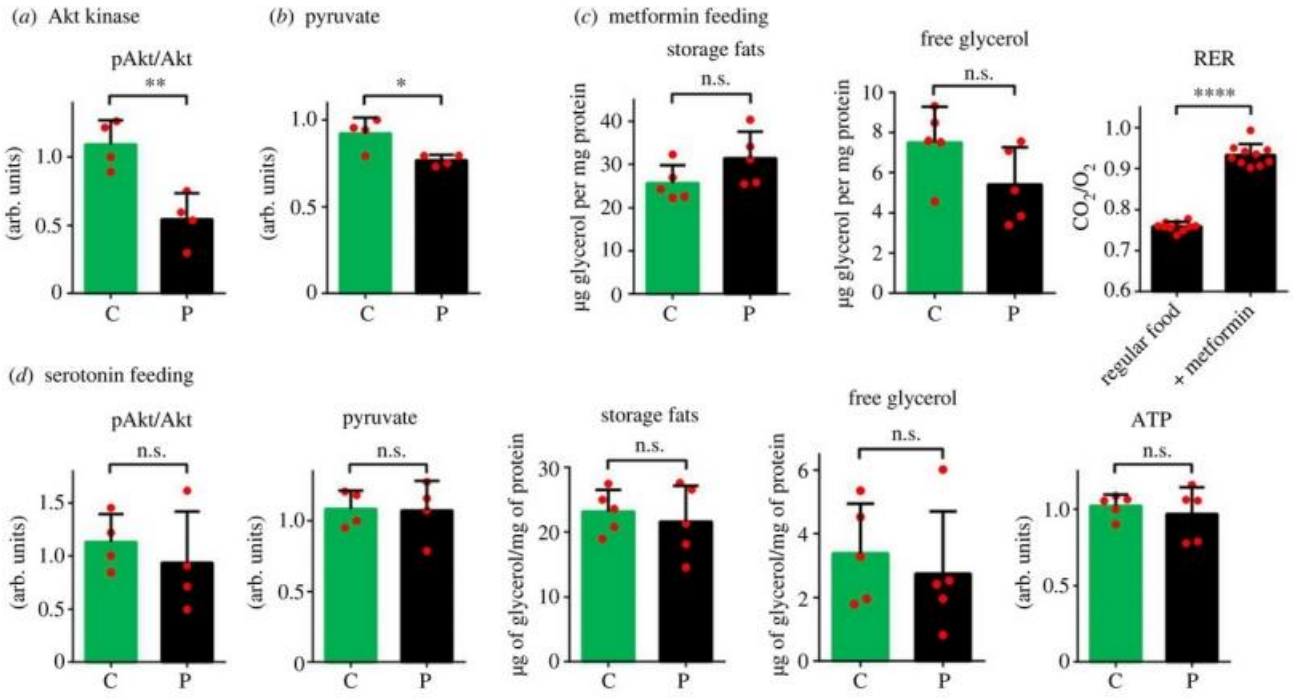

Figure 8. Effects of predator stress on metabolism/behavior and pharmacological complementation in flies reared with spiders (predators) or without (control) spiders. (a) Phosphorylation of Akt kinase at Ser505. (b) Levels of pyruvate. (c) Effect of metformin feeding on storage fats, free glycerol, and predator-reared flies' respiration exchange ratio. (d) Effects of serotonin feeding on Akt phosphorylation, pyruvate, storage fats, free glycerol, ATP and predator-reared flies' RER. In all cases: *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, n.s.—not significant. C—control, P—predator-reared.

3.7 Serotonin complements metabolic dysfunction (III)

The responses to external stimuli leading to different stress conditions are often mediated by changes in neurotransmitter levels. Serotonin dysregulation has been specifically associated with neurological stress that can cause several types of disorders in humans. In fact, w^{1118} strain flies with a mutation in the *white* gene and severely reduced serotonin levels compared with red-eyed strains (Borycz et al., 2008; Sitaraman et al., 2008) displayed a much stronger metabolic shift ($n = 10$). This could mean that serotonin mediates the effects of predator stress downstream from other parts of fly metabolism. We therefore asked whether elevated serotonin can alleviate

predator-induced metabolic impairment. We fed flies with elevated concentrations of the serotonin precursor and analyzed its effects on Akt phosphorylation (n = 8), pyruvate (n = 8), triglycerides (n = 10), free glycerol (n = 10), ATP (n = 10) and RER (n = 20) (Fig. 8d). In all cases, external administration of serotonin precursor restored these parameters in spider-reared flies to control levels, suggesting that supporting serotonin synthesis is sufficient for countering these metabolic alterations.

3.8 Effects of predator-induced stress on movement activity (III, IV)

We observed a rapid decrease in the activity of flies reared with spiders (Fig. 9).

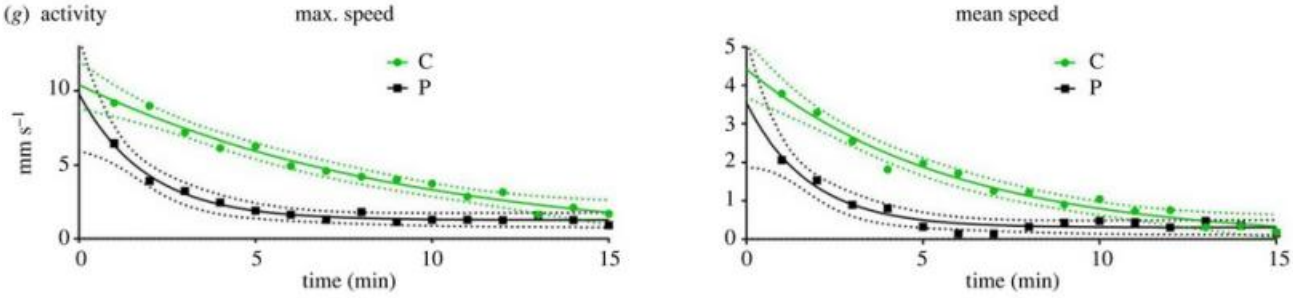

Figure 9. Nonlinear regression of maximum speed and mean speed measured across 15 min; test *P*-values for both cases are below 0.0001. Dots represent averages of 16 (control) and 23 (predator) experiments. The *P*-values of independent samples *t*-tests are 0.00017 for mean and 0.0047 for maximum speed. Dashed lines represent 95% confidence intervals.

We found significant (χ^2 =13.00, P = 0.003) differences in the distance travelled: the flies of the control group covered longer distances (5039 ± 3517 mm; mean ± SD) within a 2-h period than the flies of the experimental group (4403 ± 3443 mm) (Fig. 10A).

There were significant differences in frequency of entering the High Acceleration State χ^2 =53.376, P < 0.001), and in Maximum Acceleration (χ^2 =119.82, P< 0.001) between the groups. Flies of the control group entered the High Acceleration State less often (4781 ± 1474 times; mean

± SD) than fruit flies raised with spiders (5746 ± 1823 times) (Fig. 10B). The flies of the control group exhibited lower speed during accelerations (7.807 ± 5.665 mm/s^2; mean ± SD) than flies grown with spiders (9.829 ± 8.086 mm/s^2) (Fig. 10C).

The control group had Motion Without Movement significantly less often (χ^2= 19.183, P < 0.001) (828 ± 476 times; mean ± SD) than the group raised with spiders (1005 ± 654 times) (Fig. 10D). This shows that flies raised with spiders exhibited more "stomping in place" movements.

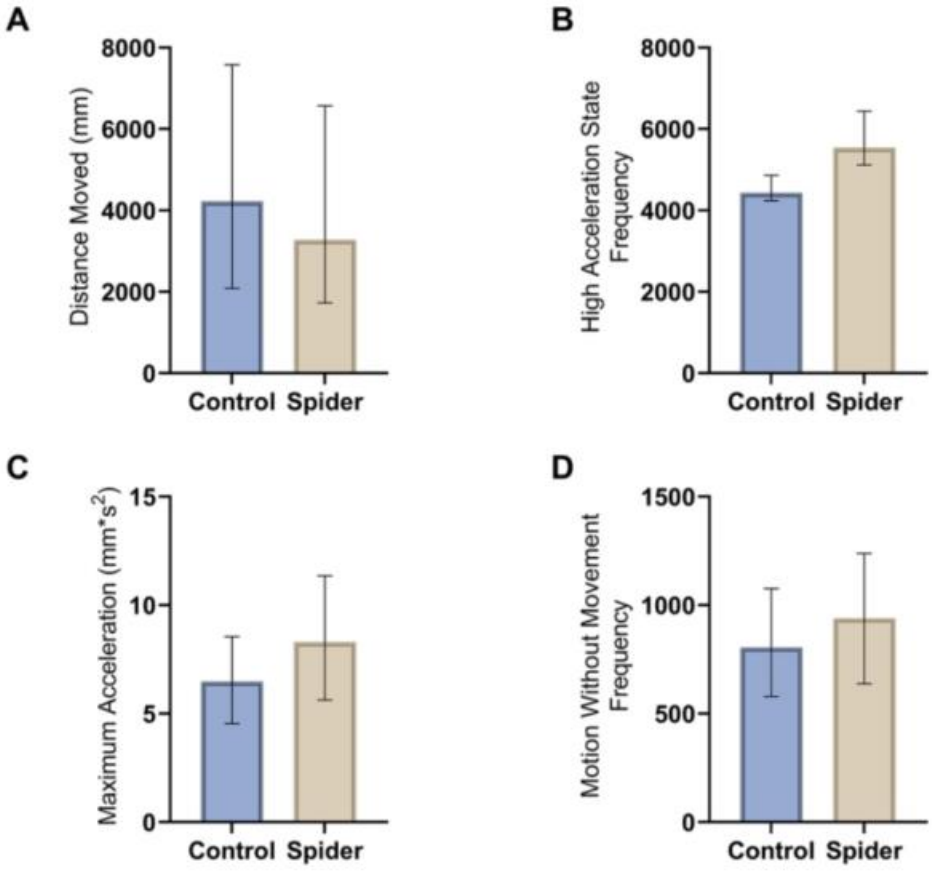

Figure 10. The median distance covered by the control group and flies grown with spiders within the 2-h period; the difference is significant at P < 0.003 (A). Difference between control flies and flies grown with spiders in the occurrence of Frequency of High Acceleration State; the difference is significant at P < 0.001 (B). Difference between control group and flies grown with spiders in the values of Maximum Acceleration; the difference is significant at P <0.001 (C). Frequency of Motion Without Movement in the control group and in flies grown with spiders; the difference is significant at P < 0.001 (D). Error bars are ± SD.

3.9 Survival of flies under predation (II, III, IV)

When exposing adult flies to predation for 12 h, their survival was significantly affected by predator presence during the larval development (two-way ANOVA: $F_{1,54} = 81.37$, $P < 0.0001$), drug treatment ($F_{2,54} = 14.76$, $P < 0.0001$), and an interaction of both those factors ($F_{2,54} = 12.57$, $P < 0.0001$). Significantly more flies survived if they were reared under predator presence (mean survival: 62% ± SD 11.4%, $n = 10$) compared to the control group (30 ± 9.4%, $n = 10$) (Tukey HSD: $P < 0.0001$; Fig. 11). Feeding flies reared with predators 5-HTP did not significantly affect their survival (65 ± 8.5%, $n = 10$) ($P = 0.985$; Fig. 11), while feeding αMW significantly decreased their survival (35 ± 7.1%) ($P < 0.0001$; Fig. 11). Feeding 5-HTP (32 ± 6.3%, $n = 10$) ($P = 0.998$; Fig. 11) or αMW (30 ± 15%, $n = 10$) ($P = 1.00$; Fig. 11) did not significantly affect the survival of flies of the control group.

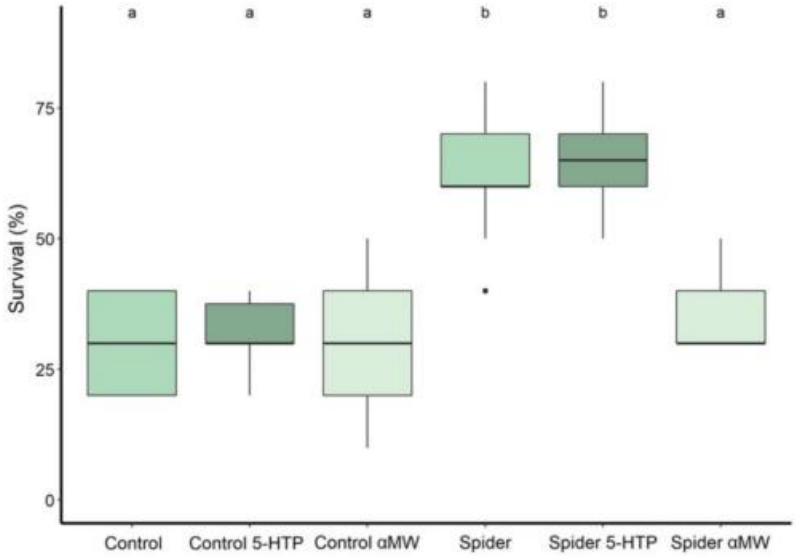

Figure 11. Survival percentage of adult fruit flies during a 12-h exposure to predation by jumping spiders. The flies reared with predators were exposed to predation during the larval stage; flies in the control group were raised without jumping spiders. Thick lines indicate the median, boxes show the Q1 and Q3 quartiles, and whiskers represent the upper and lower quartile, excluding outliers. Black dots represent outliers: data points more than 1.5 times interquartile range away from Q1 and Q3. Experimental groups that are not statistically

significantly different (Tukey HSD, $P > 0.05$) are indicated by the same letter at the top of the figure.

The changes in metabolism and decreased locomotor activity may also affect the survival of flies. We housed flies together with predatory spiders (10 male flies and 1 spider per group; 10 experimental groups in total) in a closed space and observed the survivability of flies over 12 h. There was an apparent increase in the survivability of predator-reared flies over control flies (Fig. 12). Remarkably, feeding metformin and a precursor of serotonin that reversed metabolic defects also decreased the survival of predator-reared flies to levels observed in the control group ($n = 20$). This demonstrates that the increased survival of flies in response to predator presence comes at the cost of metabolic health.

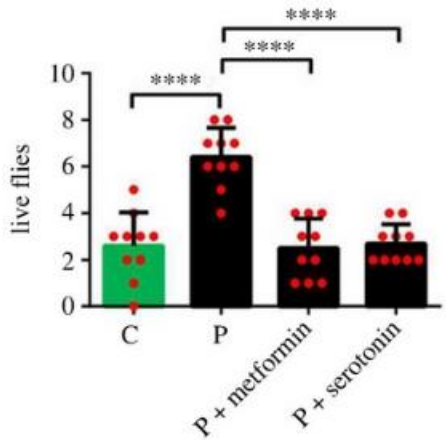

Figure 12. Survival of predator-reared and control flies with or without feeding 5-hydroxytryptophan or metformin after 12 h of incubation with spiders. In all cases: ****$P <$ 0.0001, C—control, P—predator-reared.

Lastly, following data on changes in serotonin-signaling and metabolism, we collected a vast amount of data on movement patterns and also tested the effects of these motions on fly survival.

We found that flies grown with spiders survived the 12 h experiment significantly better (χ^2 = 10.605, P = 0.0011) than naïve individuals from the control group grown without spiders during their larval stage (Fig. 13). On average, 1.6 ± 0.97 (mean ± SD) out of ten flies survived in the control group and 3.6 ± 0.97 (mean ± SD) survived in the group grown with spiders.

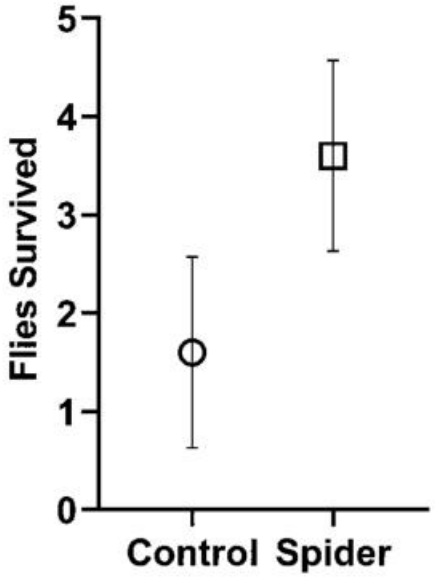

Figure 13. A mean number of ten control flies and ten flies grown with spiders surviving after a 12-h exposure to a spider. The difference is significant at P = 0.0011; Error bars are ± SD.

4. DISCUSSION

4.1 Genetic and environmental influences on phototactic variability in *D. melanogaster* (I)

In this study, we examined light-choice probability and the variation beyond expectation of the light choice probability in fruit flies from tropical and boreal climates. Although all flies were raised and tested in identical conditions, Kenyan fruit flies originated from areas with stable and predictable climate, while Finnish fruit flies originated in the northernmost limits of the species' distribution range, a zone characterized by an intensely fluctuating climate. We found that flies from the southern climate and were more strongly attracted to light (higher LCP), and more consistent across individuals (lower VBE) than flies from the northern climate. Conversely, flies from the higher latitude site were less attracted to light and less consistent across individuals. Bet-hedging theory predicts that higher phenotypic diversity may be adaptive under conditions of less predictable climate. Kain et al. (2015) developed a computational model suggesting that this hypothesis is plausible specifically with respect to *Drosophila* light-choice behavior. The results of this study provide empirical evidence in support of this model using fly strains caught at geographic sites with differential climatic variability (Akhund-Zade et al., 2020).

Previous work shows that genetic variation within lab strains likely cannot account for the variation of phototactic responses (Kain et al., 2012). In contrast, VBE varied significantly between flies collected in eastern Africa and northern Europe, suggesting that genetic factors underlie differences in the magnitudes of variation. A genetic basis for variability has been found in several other *Drosophila* behaviors including locomotor bias (Ayroles et al., 2015) and odor preference (Honegger et al., 2020). With respect to phototaxis, it was found that the gene *white* has an important role as an importer of metabolic precursors of serotonin (Kain et al., 2012). In this study, we significantly decreased VBE of Kenyan flies by feeding them αMW. Feeding Finnish fruit flies 5-HTP, a serotonin biosynthesis precursor, significantly reduced their VBE. Thus, manipulations to reduce serotonin levels in Kenyan flies and increase them in Finnish flies made VBE of these groups statistically similar. Notably, feeding Kenyan flies 5-HTP did not significantly reduce the VBE of these flies. Likewise, feeding Finnish flies αMW did not increase their VBE. These results suggest that a possible ceiling effect in Kenyan flies renders them insensitive to additional serotonin. Conversely a floor effect in Finnish flies may explain their

insensitivity to serotonin inhibitors (Lam et al., 1996). However, we cannot exclude the possibility that the 5-HT-related effects on light choice and the variability of choices are also due to independent mechanisms. For example, independent genetic effects on the mean and variability of light choice were observed when the *white* gene was muted (Kain et al., 2012).

We found that Finnish flies exhibited high VBE for phototaxis, similarly to what was observed in a laboratory strain, *D. melanogaster* w^{1118} (Kain et al., 2012). These flies have white eyes (Green, 1996; Morgan, 1910) due to a mutation in the gene *white*, which is a central part of the eye-pigmentation pathway (Ferreiro et al., 2018). This gene encodes *white*, an ATP binding cassette transporter (Pepling & Mount, 1990), that heterodimerizes with either Brown or Scarlet proteins, encoded by *brown* and *scarlet* genes to transport guanine or 5-HTP, respectively. In neurons, these transporters contribute to the biosynthesis of amines. It has been shown that *white* mutants have significantly reduced levels of the neurotransmitters serotonin (up to five times lower), dopamine, and histamine (Borycz et al., 2008; Sitaraman et al., 2008), especially in glia and neurons of the brain (Borycz et al., 2008). These diminished concentrations of the neurotransmitters in *white* mutants (Borycz et al., 2008; Sitaraman et al., 2008) have multiple consequences on a variety of neurological phenotypes affecting male courtship behavior (Anaka et al., 2008; Lee et al., 2008; Zhang & Odenwald, 1995), anesthesia resistance (Campbell & Nash, 2001), aggressive behavior (Hoyer et al., 2008), spatial learning and olfactory learning (Anaka et al., 2008; Diegelmann et al., 2006; Sitaraman et al., 2008), duration of periods of locomotion recovery following anoxia (Xiao & Robertson, 2016), sensitivity to ethanol (Chan et al., 2014), sensitivity to certain tactile stimuli (Titlow et al., 2014) and propensity to retinal degeneration (Ferreiro et al., 2018). Although Finnish fruit flies have normal red eyes, they displayed average light preference (68%) and VBE (0.72) similar to the values seen in *white* mutants. For example, w^{1118} mutants chose light 61% of the time and their VBE is ~0.87, values which are closer to those in Finnish flies than, for example, flies of the standard lab wild type strain Canton-S (76% and 0.56, respectively; Kain et al., 2012).

Common factors may be responsible for the behavioral metrics of Finnish and w^{1118} flies. We found that 5-HTP significantly affected VBE of Finnish flies, while it had no effect on VBE of Kenyan flies. The same pattern was observed in w^{1118} and Canton-S flies, respectively (Kain et al., 2012). This suggests that the brains of African flies contain a higher concentration of serotonin,

perhaps because their food sources are more diverse and may contain more metabolite precursors than Finnish flies. Tryptophan, a serotonin precursor, is an essential amino acid because animals cannot synthesize it but instead must obtain it through their diet. While African flies often enjoy the availability of different fruits, mushrooms, sap fluxes year-round, Finnish fruit flies have much shorter summer season in general, with reduced availability of rotting and decaying fruits and mushrooms in particular (Sardeshpande & Shackleton, 2019). The depletion of tryptophan from the diet has been used to assess brain serotonergic function in humans (Lam et al., 1996). This procedure is capable of rapidly lowering brain tryptophan levels in human patients by over 80% within just a few hours (Young et al., 1985), which may have immediate effects on depression patients (Neumeister et al., 1998; Smith et al., 1997) such as deviations from normal behavior and lowered food intake (Rantala et al., 2018, 2019).

Theory predicts that the relative stability of the local climate in Kenya should favor heritable and lower variability phototactic preferences, i.e., a strategy with less stochastic bet-hedging (Hopper, 1999). In such strategies, the current mean phenotype always lags environmental fluctuations, because evolution by natural selection is not instantaneous. In predictable environments, the penalty for this lag is minimized. By contrast, Finnish flies showed significantly more variable phototactic preferences, suggestive of an adaptive bet-hedging and consistent with previous modeling of bet-hedging in thermal preference behavior (Kain et al., 2015). Interestingly, adaptations for heat resistance have the potential to improve cold resistance (Condon et al., 2014). This shows that adaptations to extreme temperatures improve not only the ability to withstand a particular deviation from mean temperatures, but also the magnitude of temperature variation. Moreover, the ability to tolerate extreme temperatures is improved in populations that evolve in fluctuating environments relative to when populations are exposed to a stable increase of high temperatures (Condon et al., 2014; Tobler et al., 2015). The high VBE of Finnish flies, which in the wild may result in a variety of thermal experiences, may serve as parallel adaptation to life in relatively unpredictable thermal and visual environments, leading flies to find conspecifics, breed and oviposit in a variety of conditions, rather than wait for specific optimal conditions that might not arrive in a particular season.

In a population utilizing a bet-hedging strategy, individuals exhibiting a wide variety of preferences are born continuously across a season. If the summer is cooler, spring-adapted

individuals will survive, while summer-adapted flies will survive if the summer is hot and long (Bergland et al., 2014). Kawecki (2000) has suggested that the phenotypic expression of genetic variation can be suppressed, and heritability reduced under fluctuating selection. Dynamic modulation of variability-suppressing serotonin is a potential mechanism to tune the canalization of the phototactic phenotype. To test this possibility, one could measure VBE and 5-HT concentration in the brains of flies, born during hot and cool summers near the northernmost areas of their distribution ranges. Our results suggest that plastic responses to environmental differences, which is another major strategy for dealing with environmental heterogeneity, is not a likely explanation for the observed differences between African and European flies. The flies of both populations were grown under identical conditions and we are not aware of any environmental fluctuations to which a plasticity strategy could respond.

While Kain et al. (2012) observed significant effects of 5-HTP on the VBE of flies, this treatment did not show any influence on light-choice probability in their study. However, we found significant effects of feeding 5-HTP and αMW on LCP, which depended on the origin of the strain. In addition, we observed effects on LCP of line origin, with Kenyan flies ~10% more photopositive. In Finnish flies, feeding 5-HTP did not affect LCP, while feeding αMW significantly reduced it, which was the opposite of what we observed in the case of VBE in these flies. Feeding 5-HTP significantly lowered LCP and feeding αMW significantly raised LCP in Kenyan flies. Thus, 5-HTP decreased the light choice probability in Kenyan flies and did not affect it in Finnish flies. Importantly, Kenyan flies on control media chose the light more often than Finnish flies on control media. Kenyan flies are likely to have a higher concentration of 5-HT in the brains, at least when fed natural diets.

It is possible that differential levels of serotonin do not explain the mean LCP of these strains, since serotonin or its precursor 5-HTP have been previously reported to decrease photopositivity in larval bryozoans (Pires & Woollacott, 1997). There may also be genetic background by serotonin-exposure effects. However, dopamine was previously reported to increase light choice (Pires & Woollacott, 1997). *White* mutants have reduced concentrations of dopamine in the brain (Borycz et al., 2008; Sitaraman et al., 2008) and if the neuromodulatory state of Finnish flies mirrors that of *white* mutants, they may also have lower dopamine levels. This in turn might explain their lower LCP, while lower serotonin could explain their higher VBE.

Kain et al. (2012) did not find any effect of dopamine drugs on VBE or LCP of different strains of fruit flies including *white* mutants. However, it has been shown that dopamine affects the production and release of melatonin (Gonzalez et al., 2020), a key driver of biological rhythm (Arendt & Skene, 2005). Melatonin production might be disrupted in the brains of Finnish flies to ensure activity during long summer days at high latitudes indicating that dopamine of boreal fruit flies, especially the receptor subtypes and the density of receptors deserve a special attention in future research.

Importantly, 5-HT is a precursor of melatonin (Richter et al., 2000), and 5-HT is also regarded as a substance affecting physiological rhythms according to the light–dark cycle in invertebrates (Hardeland & Poeggeler, 2003). Kenyan and Finnish flies likely have different diurnal rhythms and sleep patterns: While there is a relatively regular day/night cycle in the tropical zone, Finnish flies enjoy never-ending daylight for up to two months at high latitudes. This may affect their serotonergic neural regulation because melatonin may be in low demand and not metabolized much during the northern summer, perhaps allowing the accumulation of elevated 5-HT in neural tissues in summer. Besides a leading role of melatonin in the determination of sleep/wake cycles, it is also a potent antioxidant with a proposed role in immune function in invertebrates (Tan et al., 2010). The suppression of nocturnal production of melatonin has detrimental effects on antioxidant systems of organisms (Jones et al., 2015) which may facilitate the bet-hedging strategy of invasive species at high latitudes.

Flies that follow a bet-hedging strategy might only attain environmental conditions well-matched to their behavioral biases if they live through long periods of poorly matched conditions. Thus, there is likely an interplay between generation/lifespan length and the timescale of environmental fluctuations. Indeed, modeling suggests that bet-hedging is an adaptive response to environmental fluctuations at specific timescales roughly corresponding to the lifespan (Krams et al., 2020). Melatonin is an antioxidant, and may lengthen the lifespan of flies (Teran et al., 2012). Thus, it has the potential to affect evolutionary behavioral strategies both directly through the neuromodulatory state, but also indirectly through an effect on lifespan. These hypotheses call for precise measurements of 5-HT, melatonin, dopamine and behavior in fruit flies across the season and the south-north gradient of their distribution range.

4.2. Serotoninergic regulation of predator-evasion tactics (II)

The presence of predators is known to alter prey morphology (Hossie et al., 2010; McCollum & Leimberger, 1997) and exert selective pressure on prey escape ability (Janssens & Stoks, 2018; Krams et al., 2016; O'Steen et al., 2002). In this study, we found that the turning choices of fruit flies grown with predators are less predictable than those of flies grown in a predator-free environment. We also show that flies raised with predators survived under predation by spiders significantly better than flies grown without predators. Our results suggest that the higher variability/lower predictability of turning behavior of flies grown with predators may make them better at evading predation. We also show with pharmacological experiments that the effects of predator-rearing on turning variability and survival of *D. melanogaster* are regulated by the neurotransmitter serotonin, which also regulates the variability of turning behavior (de Bivort et al., 2022). However, these serotonin-associated effects applied only for fruit flies grown with spiders.

Unpredictable and erratic turning behavior in some animals makes them more challenging to attack (Bilecenoğlu, 2005; Eifler & Eifler, 2014; Yager et al., 1990), as is seen in both experimental (Jones et al., 2011) and modeling (Richardson et al., 2018) studies. Individual insects can exhibit substantial differences in escape behaviors, even in the absence of genetic variation (Schuett et al., 2011). Our results suggest a link between less predictable turning behavior and better survival under predation risk by jumping spiders that are sit-and-wait predators. One explanation is that growing up with predators provides prey with signals that are not generated by transient contact with predators' post-development. Perhaps the effect of these signals is mediated by serotonergic neuromodulation during prey development. This idea is consistent with the observation that flies fed αMW during development, but without predators present, showed similar adult survival in the presence of spiders as control flies, suggesting that fruit fly individuality is not solely driven by 5-HT (Maloney, 2021).

Some previous work has shown that fruit flies reared in identical lab environments show broad diversity in their phototactic choices, variability which is under the control of 5-HT (Kain et al., 2012; Krams et al., 2021). Notably, inhibiting 5-HT synthesis was associated with higher phototactic variability — here we observed that inhibiting 5-HT reduced the excess turn bias variability seen in flies reared with spiders. Geographic variation of fruit fly phototaxis was

consistent with a negative relationship between 5-HT and variability of phototactic choices. Flies from northern climates grow on food relatively deficient in the metabolic precursors of serotonin and had lower predictability of phototactic choices (Krams et al., 2021). Thus, the association between 5-HT and behavioral predictability went in opposite directions in the present study and previous work examining phototaxis. These contradictory results suggest that the control of 5-HT over different behaviors may lead to different results, probably because different serotonin-responsive neuronal circuits are involved in different behaviors. To better understand the developmental, epigenetic and neurophysiological changes caused by direct predation and non-lethal predator presence, more study of behavior-specific neurobiological effects is required.

Our results support the results by Pantoja et al. (2016) examining variability in zebrafish (*Danio rerio*) antipredator locomotor behaviors. They found that zebrafish individuals show significant variation in acoustic startle responses. These responses are linked with the neurosecretion of dorsal raphe neurons (Pantoja et al., 2016). It was shown that zebrafish individuals show a higher fraction of serotonergic dorsal raphe nucleus neurons active during predator attacks. Pantoja et al. (2016) also showed that heightened 5-HT prevented habituation to predator stimuli, which improves the efficiency of antipredator behavior and survival of the prey. Together, these results suggest the importance of serotonergic signaling in the CNS and its ontogenetic development in establishing a distribution of antipredator behaviors across individuals.

The results of this study may have evolutionary implications. It is known that without phenotypic variation, there would be no evolution by natural selection. However, we show that individuals with similar genotypes raised in similar environments, except for the presence/absence of spiders, may significantly differ in their simple behavioral reactions, (such as left vs. right decision in the absence of an asymmetric stimulus in the Y-maze). This suggests that asymmetries within the brain predispose the animal to go one way rather than the other and that neural activity influences the variation between animals (Buchanan et al., 2015). As these predispositions are relatively stable within individuals with considerable among-individual differences in behaviors (Buchanan et al., 2015; Reale et al., 2010; Roche et al., 2016; Trakimas et al., 2019), behavioral reactions of this kind are coined animal personality. Our results show that fruit flies may use a simple mechanism to dynamically regulate their behavioral individuality with individual variation

in wiring and behavior as a general feature of neural circuits to facilitate individual adaptations and survive in changing environments (Mollá-Albaladejo & Sánchez-Alcañiz, 2021). However, explaining the proximate origins of changes in behavioral variability as a response to environmental challenges is not easy. Behavioral phenotypes emerge from many different levels of biological organization, including sensing of predators in the environment, adaptive gene expression, and even stochasticity in gene expression (Honegger & de Bivort, 2018; Li et al., 2017; Raj et al., 2010) to develop biases in idiosyncratic behavioral responses (Werkhoven et al., 2021) without changes in average left-right turning preferences.

This study found that flies reared with spiders were less mobile than control flies. Our recent study shows that predator stress during larval development of *Drosophila* impairs carbohydrate metabolism by systemic inhibition of Akt protein kinase, which is a central regulator of glucose uptake (Krama et al., 2023). This metabolic disorder is a likely cause of developing a diabetes-like biochemical and behavioral phenotype. An inability to metabolize glucose shifts the metabolism of fruit flies to triglyceride consumption, which decreases walking activity and might be a direct reason for the enhanced survival of fruit flies grown with spiders. Consistent with this idea, carbohydrate metabolism was found as one of the molecular functions most enriched in genes whose expression variation predicts variation in locomotor activity among individual isogenic flies (Werkhoven et al., 2021). However, the mechanism causing the higher variability of the turning behavior in flies with a diabetes-like phenotype remains unknown.

Antipredator behavior consists of a complex set of behavioral and physiological reactions and therefore likely involves neural pathways other than 5-HT. Honegger et al. (2020) found that both 5-HT and dopamine affect olfactory preference variability in fruit flies, and it is known that fruit flies can detect predators by their odors (Krams et al., 2021). Omura et al. (2012) and Stern et al. (2017) showed that the roaming speed of animals might depend on such neurotransmitters as tyramine, octopamine, npr-1, and daf-7, in addition to 5-HT. This suggests that future research on the neural regulation of antipredator responses in fruit flies should examine the effects of several neurotransmitters and their possible interactions. Experimental manipulations targeting more than one neuromodulator may be essential, as one neuromodulator can alter the efficacy of other neuromodulators (Niederkofler et al., 2015; Niens et al., 2017). Finally, animals may respond to neuromodulators differentially based on their personalities (Krams et al., 2018). The complex

interactions of neuromodulators and their behavior-specific effects on predictability will make this a rich and challenging area of research.

4.3. Metabolic and behavioral adaptations to predator stress (III)

The effects predators have on prey are not limited to the death of prey individuals, but can induce a lasting condition of fear in the prey that survive in the presence of predators. As a result, prey often respond to predators in their environment by altering their morphological and physiological phenotypes during development (Hawlena & Schmitz, 2010a, 2010b; Hossie et al., 2010; I. Krams et al., 2016; Lehmann et al., 2014). Although these changes facilitate survival by improving escape abilities (Janssens & Stoks, 2014; I. Krams, 2002), predators may have enduring costly effects on prey individuals (Siepielski et al., 2014; Zanette & Clinchy, 2020). For example, predator-induced fear is one of the most common stressors employed in animal model studies of post-traumatic stress disorder (Zanette et al., 2019). This research has gained scientific interest because of the relevance of psychological stress in causing clinical depression and other metabolic disorders, such as type 2 diabetes, in humans. Although the underlying mechanism has remained unclear, increased serum glucocorticoid concentrations and catecholamine release are commonly associated with the development of insulin resistance (Beaupere et al., 2021). Our results align with these findings by showing that *Drosophila* reared with predators develop a diabetes-like biochemical phenotype characterized by an inability to metabolize glucose, forcing a shift to triglyceride consumption. This is caused by a decreased activity of Akt kinase, a central regulatory kinase that has a major role in controlling glucose uptake. This protein facilitates a highly conserved glucose transport mechanism (e.g., via GLUT4-dependent pathway in muscles), and defects in this pathway are therefore closely associated with the development of diabetes (Huang et al., 2018). Improving glucose transport using metformin, which has similar effects in flies to those in humans (Bahhir et al., 2019), restored the original metabolic balance in flies grown with predators.

Predator presence eventually changes the quality of the environment and affects the survival strategies of prey. While *Drosophila* flies rely on visual and olfactory cues for detecting predators such as spiders and mantises, it is currently unclear to what extent flies use separate sensory systems in different environmental conditions (Flor et al., 2017; I. Krams et al., 2016).

However, they do have a highly developed olfactory system that allows them to live for generations in complete darkness (Izutsu et al., 2016). This sensory system is sufficient by itself to detect the presence of spiders, and even exposure to spider odors can elicit metabolic and developmental changes (Krams et al., 2016; Krams et al., 2021). Regarding w^{1118} flies, it is also unclear whether they can use vision to detect predators, as they may have poor visual acuity (Kalmus, 1943). However, w^{1118} flies easily chose walking corridors when tested in the Y-maze experiments (Buchanan et al., 2015), suggesting w^{1118} flies actively rely on vision while exploring their environment.

Although a number of studies have described how chronic stress can have enduring effects on metabolism and behavior (Chen et al., 2014; Wu et al., 2019; Zanette et al., 2019), the connection between neural chemistry and metabolism has remained unclear. Our finding that supporting serotonin synthesis antagonizes the described metabolic effects suggests a central role for serotonin in such biochemical communication. Serotonin has multiple biological functions: regulating courtship behavior (Zhang & Odenwald, 1995), affecting spatial memory (Diegelmann et al., 2006) and olfactory learning (Anaka et al., 2008), influencing phototactic behavior (Kain et al., 2012; Krams et al., 2021), and affecting turning behavior (Krama, et al., 2023). Furthermore, it participates in several pathways that overlap with the roles of other neurotransmitters, such as dopamine and octopamine (norepinephrine homologue in *Drosophila*). Owing to the variety of serotonin's roles in neural circuits, which are at least partially redundant, its effect on metabolism can be caused by a number of different mechanisms. One plausible explanation could be related to the observed interconnection between serotonergic and insulin-producing nervous systems. In *Drosophila*, serotonergic neurons are closely apposed with insulin-producing neurons, and these two neuronal systems communicate (Kaplan et al., 2008). They control insulin signaling and, if defective, serotonin and insulin accumulate together, with suppressed peripheral insulin sensitivity. In humans, elevating serotonin has beneficial effects on metabolic balance, improving insulin sensitivity and glucose homeostasis (Al-Zoairy et al., 2017). This effect is relayed through serotonylation of the small GTPase Rab4, which elicits beneficial effects on glucose uptake, thus representing a convergence point between serotonin and insulin signaling. Since serotonin is decreased in human psychological disorders resembling the effects of predator stress, it is tempting to speculate a linear relationship between the metabolic reprogramming described here and serotonin levels. Tentative support for this hypothesis comes from a quantitatively stronger

metabolic shift in the serotonin-depleted w^{1118} strain. However, serotonergic upregulation caused by the exogenous administration of serotonin might also elicit the observed reversion of metabolic changes.

Systemic effects on catabolism in predator-stressed flies resembled the effect of glucocorticoids in humans, a group of hormones released in response to stress conditions through activation of the HPA axis. These hormones antagonize the function of insulin by inhibiting the uptake of glucose in muscles and adipose tissue. They also downregulate glycolysis, inducing lipolysis and hepatic gluconeogenesis (Kuo et al., 2015). This mobilizes and reroutes energy reserves for specific tasks, e.g. increasing blood glucose levels to prepare the organism for a 'flight-or-fight' response.

Drosophila has no apparent neuroanatomical homologue of the mammalian HPA axis nor the same glucocorticoid hormones as humans. However, it has a central steroid hormone ecdysone, converted into 20-hydroxyecdysone (20HE) in hemolymph after its release. Best known for its role in inducing larval mounts and metamorphosis (Yamanaka et al., 2013), it also regulates metabolism by suppressing glucose use. Binding with its receptor (EcR) induces this protein's translocation to the nucleus, where it represses the transcription of genes central to glucose utilization (Kovalenko et al., 2019). This is antagonistic to the function of an oestrogen-like receptor (ERR) recently described as a receptor for glucocorticoids in *Drosophila*, suggesting an interplay with other steroid hormones in this organism (Bartolo et al., 2020; Tennessen et al., 2011). The effects of 20HE are very similar to the deletion of ERR, which blocks the use of carbohydrates as a fuel source, leading to a shift towards lipid oxidation and depleting triglyceride reserves (Tennessen et al., 2011). Furthermore, 20HE acts as a stress hormone in flies, upregulated in response to adverse environmental conditions and stressful social interactions (Ishimoto et al., 2009).

Intriguingly, we found that predator stress enhances the survival of spider-reared flies in the adult stage when kept together with the spiders. This effect correlated precisely with metabolic reprogramming since the administration of metformin and serotonin precursor reverted the survival advantage to control levels. This indicates that this metabolic reprogramming is adaptive and provides a clear survival benefit at the expense of reduced metabolic fitness. One explanation for this finding is associated with the speed of movement and overall locomotor activity of the

flies. Another possibility is linked to the glucocorticoid stress effect on memory. Stress-induced glucocorticoid release enhances memory consolidation and long-term memory in humans (Roozendaal, 2002). The effect is the same in flies, with ecdysone having a clear beneficial impact on long-term memory formation (Ishimoto et al., 2009, 2013). It is believed that these effects of glucocorticoids are linked to the conservation of glucose for neural tissue function, which is a primary carbon source. This adaptation fuels increased neural activity, especially learning and memory (Wirth, 2015). Brains are metabolically costly organs, as is the process of creating new memories (e.g., via increased synaptic connections) (Burns et al., 2010). In fact, elevated carbohydrate uptake in humans and animals, including *Drosophila*, has an apparent memory-enhancing effect, especially for long-term memory (Greenwood & Winocur, 2001; Placais et al., 2017; Smith et al., 2011; Totani et al., 2020; Winocur & Gagnon, 1998).

In the aggregate, the results of this study allow us to propose a model explaining how chronic psychological stress, such as predator stress, induces metabolic disorders. Shunting glucose away from catabolically active tissues like muscle to be consumed by neurons is likely an adaptation to create memories and prepare for similar stressful conditions in the future. However, when stress persists and leads to chronic activation of the HPA axis and sustained glucocorticoid release, it will impair normal glucose metabolism and permanently shift systemic catabolism towards lipid oxidation, preventing the use of carbohydrates. Such loss of metabolic flexibility, especially in animals that use carbohydrates as the main form of energy source, will inflict fitness costs, leading to decreased ATP production and downstream effects on resistance to nutritional scarcity and locomotor activity. Therefore, this chronic activation of a mechanism that provides short-term benefits will cause decreased fitness if stress persists. This is supported by the observation that chronic activation of ecdysone signaling, although beneficial for an immediate response, can cause negative long-term effects (Ishimoto & Kitamoto, 2011).

The results of this study suggest that although the diabetes-like phenotype induced by predator presence reduces general health, it might be beneficial for survival. The insulin-producing system in *Drosophila* and other invertebrates differs to some extent from that of vertebrates, including humans. *Drosophila* flies have eight insulin-like peptides (Wu & Brown, 2006), which likely have different and partially overlapping roles in metabolism regulation (Gronke et al., 2010). This shows that insects may have numerous ligands for one receptor, while mammals have

receptors with somewhat redundant functions but a restricted number of ligands. Also, while the effect of extra 5-HTP in increasing serotonin is straightforward, it might affect concentrations of another neurotransmitter. Tryptophan is a precursor of biopterin (Joh, 2000), a cofactor associated with serotonin and dopamine synthesis. While the metabolic shift in serotonin-depleted w^{1118} flies compared with the Oregon strain provides tentative support for decreased serotonin concentration in response to predator stress, neuron-specific measurements are required to fully understand the mechanism underlying this hypothetically adaptive metabolic shift.

Finally, metabolic disorders are often associated with the impairment and loss of dopaminergic function (Bell et al., 2020). Predator-induced stress affects the levels of brain dopamine, which are decreased in rats exposed to predator stress (Dremencov et al., 2019; Kondashevskaya et al., 2022). Since the w^{1118} strain has reduced dopaminergic activity, the interconnected serotonin and dopamine pathways should be studied simultaneously in predator-induced stress. These numerous aspects must be considered to fully understand the role of stress in the development of metabolic phenotypes and similarities/dissimilarities of stress perception in humans and *Drosophila* (Graham & Pick, 2017).

4.4. Behavioral variability and metabolic disorders as responses to predator stress (IV)

In this study, nearly 70 million data points were collected for *D. melanogaster* not exposed to spider presence during their larval development (n = 839) and those flies (n = 729) subjected to predation stress during their larval stage using a high-throughput data sampling method (Kain et al., 2012; Krama et al., 2023). Short-term stress promotes oxidative stress and changes the metabolic balance away from anabolism and high-molecular-mass compound production, resulting in increased glycogen generation and hence a more significant requirement for carbohydrate intake (Trakimas et al., 2019). However, chronic psychological stress differs from short-term acute stress because prolonged stress, such as predator stress, may induce metabolic disorders (Krama et al., 2023). As a result, the stress of encountering a predator early in life may alter an adult organism's phenotypic appearance, behavior, and metabolism. Our results support earlier findings that walking activity is reduced in flies grown with spiders; a possible explanation

for this is because diabetes-like metabolic disorder prevents fruit flies from using carbohydrates and shifts catabolism toward fat utilization (Krama et al., 2023). Therefore, oxidation of lipids is expected to contribute proportionally more to major metabolic functions, including walking and flight movements in fruit flies grown with spiders than in control flies. Although fats are the most energy-rich macronutrient, fatty acids are a slower energy source than carbohydrates, requiring oxidative phopshorylation to generate ATP (Brosnan, 1999). We show that fruit flies raised with spiders walk less while their initial movement acceleration is higher than in the control group, suggesting a more rapid exhaustion in flies grown with spiders.

In this study, we also confirmed that flies grown with spiders survived better in adulthood under direct exposure to predation risk than those from the control group grown without any previous contact with predators. The flies affected by predation risk were observed to move in frequent and short dashes. Importantly, their initial speed (acceleration) was substantially higher than that of flies of the control group. We found that fruit flies from the control group moved at a more measured pace characterized by rare and low-intensity accelerations. Thus, the two groups of fruit flies radically differed in their movement pattern. Interestingly, during their rest stops, fruit flies reared with spiders moved their bodies (stomped in place) more often, which was found using the Motion Without Movement parameter. Thus, fast accelerations, less distance walked, and distinctive "stomping in place during rest" behavior may make fruit flies grown with spiders sooner to leave dangerous areas and become less attractive to spiders while resting between two subsequent walks.

The swift and sporadic stomping in place is a kind of unexpected behavior of fruit flies grown with spiders. Instead of efficiently accumulating energy for the next series of walks, these flies spend their rest while quickly moving/shaking their bodies without spatial displacement. Despite being potentially more conspicuous to predators because of this activity, flies grown with spiders survived better than control flies when exposed to predators as adults. One explanation for this is that by turning in place and making small movements while staying in the same spot, these flies give predators false signals of their immediate future activities, such as flight initiation behavior (Card & Dickinson, 2008).

Another explanation for the improved survival of flies reared with spiders is that the exposure of fruit flies to predators may cause metabolic disorders, and active motions without

spatial displacement may reflect conditions of altered physiology, such as sickness behavior characterized by a variety of coordinated symptoms such as anxiety, chaotic grooming behavior, and failure to concentrate (Hart, 1988). It has been traditionally considered that predators are supposed to select substandard prey such as young, inexperienced, or sick individuals (Genovart et al., 2010). However, it has also been shown that some predators can non-randomly avoid infected prey (Hamilton & Zuk, 1982; Jones et al., 2005; Meyling & Pell, 2006). Although this strategy of predation has received much less attention in the literature (Gutierrez et al., 2022), our results show one more mechanism for the improved survival of sick animals expressing less predictable and more erratic walking responses than fruit flies without a diabetes-like biochemical phenotype (Krama et al., 2023). Previous research showed that fruit flies with a diabetes-like biochemical phenotype rely only on fat as a catabolic fuel source, causing lower body fat content (Krams et al., 2016) and a 20% decrease in ATP levels (Krama et al., 2023). Also, fruit flies grown with spiders are known to have higher body nitrogen (N) content, suggesting increased muscle mass in these flies (Krams et al., 2016). Thus, higher body N and muscle mass, lower fat reserves, faster accelerations and faster exhaustion, more "stomping in place" behaviors, and lowered availability of ATP may explain more erratic and less predictable walking locomotion and better survival of fruit flies grown with spiders. Future research should test whether spiders actively avoid fruit flies with metabolic disorders and flies with infectious diseases and whether the behavior of infected flies resembles that of fruit flies experiencing metabolic diseases.

This study shows that some conditions other than infectious diseases can make fruit flies unpreferred prey as individuals grown under sustained stress of predation survived better than control individuals when exposed to spider predation. Encountering stress during development and adulthood may lead to metabolic disorders, such as PTSD (Zanette et al., 2019) and diabetes-like phenotypes (Krama et al., 2023), often affecting the nervous and endocrine systems. Although the link between psychological conditions and dysfunctional glucose catabolism has been established (Hackett & Steptoe, 2017), our understanding of the signaling pathways connecting environmental stress, behavior, and biochemistry is rudimentary, and little is known about the impact of environmental stress on systemic metabolism. Based on the interconnections between physiology and behavior, we would predict higher senescence rates of walking behavior in flies grown with spiders. Eventually, even young fruit flies demonstrate a shift toward inefficient energy consumption at short sprints and an incapacity to cover long distances without accessible energy

sources. Overall, a link between sickness behavior and improved survival under predation risk looks tempting; however, future research on the sickness behavior of fruit flies and other animals is needed because the underlying biochemical and behavioral mechanisms seem complex. Further studies on metabolism and movement of larvae, as well as the effects of senescence and their influence on behavior are also essential to develop a comprehensive interpretation of the observations.

CONCLUSIONS

Predation risk was shown to significantly impact the serotonin signaling pathway, leading to increased variability in turning behavior among adult fruit flies. This increased behavioral unpredictability, as measured by the Median Absolute Deviation of turning biases, suggests a dynamic regulation of animal personality traits in response to environmental stressors, in this case, predation. The findings demonstrate that exposure to predators during developmental stages enhances the survival chances of fruit flies by making their movement patterns less predictable to predators, thereby complicating predator attack strategies.

Moreover, the studies highlighted a profound impact of predation stress on the metabolic processes within *Drosophila melanogaster*. Exposure to predators induced a diabetes-like phenotype characterized by impaired carbohydrate metabolism, systemic inhibition of Akt protein kinase, and a shift towards the utilization of fatty acids over carbohydrates. This metabolic dysregulation, while detrimental to the organism's energy balance and locomotor activity under normal conditions, conferred an advantage in predator-rich environments by possibly enhancing cognitive functions and behavioral responses crucial for evading predators.

Interestingly, the administration of metformin and 5-hydroxytryptophan was found to reverse the metabolic impairments and the survival advantage under predation, suggesting that the predator-induced metabolic and behavioral changes are closely linked to serotonin signaling pathways. These interventions highlight the potential for manipulating metabolic and neurotransmitter pathways to influence behavioral outcomes and survival strategies in prey species.

The research underscores the complexity of prey responses to predation stress, revealing that the non-lethal effects of predator exposure extend beyond immediate behavioral adaptations to encompass significant biochemical and physiological alterations. These changes, while adaptive in the context of predation, underscore the intricate balance between survival strategies and metabolic health in *D. melanogaster*. The findings suggest insights into the evolutionary pressures shaping prey defenses and the potential trade-offs involved in adapting to predation risk.

ACKNOWLEDGEMENTS

I express my deep gratitude to my scientific supervisor professor Indriķis A. Krams. My work in science in general and this dissertation in particular became possible only through his tireless support and mentoring. I am also especially grateful to Tatjana Krama for careful laboratory work supervision, motivation and training.

I extend my gratitude to our co-author professor Benjamin L. de Bivort. His research and insights were key to this thesis' ideas development.

Special thanks to Giedrius Trakimas for his long and patient guidance in statistics, Colton Adams for meticulous English language proofing, and Ronalds Krams for his careful help with administrative tasks.

I would also like to thank my colleagues and co-authors Priit Jõers, Jorge Contreras-Garduño, Markus J. Rantala, Anne Must, Enno Merivee and Maris Munkevics, whose productive collaboration has allowed me to develop many skills and acquire new knowledge.

I express my gratitude to Eriks Sledevskis, Valdis Mizers, Diana Bahhir, Vadims Bartkevičs, Iveta Pugajeva and Didzis Elferts for prompt and exceptional cooperation.

My most important thanks go to my parents for their advice, constant energy and commitment, which allowed me to make it all the way to my doctoral thesis. I am grateful to my wife Alina Popova for her understanding, patience and motivation, as well as to my dog Bruschetta for providing psychological support.

The thesis was supported by European Social Fund project Nr. 8.2.2.0/20/I/003 and the Latvian Council of Science project Nr. lzp-2024/1-0437.

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ORIGINAL PAPERS

I PUBLICATION

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 16 April 2021
doi: 10.3389/fnbeh.2021.659331

Serotoninergic Modulation of Phototactic Variability Underpins a Bet-Hedging Strategy in *Drosophila melanogaster*

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OPEN ACCESS

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Specialty section:
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

Received: *27 January 2021*
Accepted: *19 March 2021*
Published: *16 April 2021*

Citation:
Krams IA, Krama T, Krams R, Trakimas G, Popovs S, Jõers P, Munkevics M, Elferts D, Rantala MJ, Makņa J and de Bivort BL (2021) Serotoninergic Modulation of Phototactic Variability Underpins a Bet-Hedging Strategy in Drosophila melanogaster. Front. Behav. Neurosci. 15:659331. doi: 10.3389/fnbeh.2021.659331

When organisms' environmental conditions vary unpredictably in time, it can be advantageous for individuals to hedge their phenotypic bets. It has been shown that a bet-hedging strategy possibly underlies the high inter-individual diversity of phototactic choice in *Drosophila melanogaster*. This study shows that fruit flies from a population living in a boreal and relatively unpredictable climate have more variable variable phototactic biases than fruit flies from a more stable tropical climate, consistent with bet-hedging theory. We experimentally show that phototactic variability of *D. melanogaster* is regulated by the neurotransmitter serotonin (5-HT), which acts as a suppressor of the variability of phototactic choices. When fed 5-HT precursor, boreal flies exhibited lower variability, and they were insensitive to 5-HT inhibitor. The opposite pattern was seen in the tropical flies. Thus, the reduction of 5-HT in fruit flies' brains may be the mechanistic basis of an adaptive bet-hedging strategy in a less predictable boreal climate.

Keywords: adaptive strategies, *Drosophila melanogaster*, phototaxis, serotonin, variation

INTRODUCTION

Life in the natural environment exposes organisms to a number of abiotic and biotic factors. The stability of environmental conditions differs across latitudes, geographic locations and time, resulting in diverse patterns of fluctuation in ecological interactions, population sizes, sex and age ratios, parasite burdens, range shifts and extinction. To predict the direction and magnitude of responses to fluctuating environments, it is important to understand the strategies that facilitate the survival of organisms under environmental change (Merilä and Hendry, 2014; Chirgwin et al., 2015; Trakimas et al., 2019).

One such strategy is phenotypic plasticity, in which organisms adjust their phenotypes in accordance with their current development conditions. Although phenotypic plasticity is a versatile strategy and can potentially apply to behavioral, morphological, physiological or biochemical traits, there are constraints to plasticity such as limits on the speed with which a phenotype can change in response to a fluctuating environment (Folguera et al., 2010; Murren et al., 2015). To survive and reproduce in fluctuating environments, individuals might use also another strategy: evolving by natural selection with the changing environment (Langerhans and DeWitt, 2004). This is one of the most powerful means to track ongoing biotic and abiotic fluctuations such as climate change.

Some organisms live in conditions where ambient temperature, precipitation, food resources, and other important selection factors occur in highly variable patterns (Siepielski et al., 2017). In these climates, the time windows favorable for reproductive cycles and offspring development can be short. Therefore, there is a pressure on individuals to start the reproductive season as soon as suitable conditions arrive. However, if all the individuals invest in reproduction at the first possible moment, the entire population is vulnerable to extinction if the favorable conditions unexpectedly cease. Therefore, it can be useful if individuals in a population, hedge their bets and some of them delay the onset of reproduction, otherwise no offspring result from the population experiencing these false starts. There are three bet-hedging strategies: Conservative bet-hedging (always play it safe), diversified bet-hedging (do not put all eggs in one basket) and adaptive coin flipping (random selection of the available strategies; Olofsson et al., 2009). Bet-hedging is potentially adaptive for many additional traits, including behavior, when the selective pressures of the environment fluctuates strongly (Kain et al., 2015).

The fruit fly *Drosophila melanogaster* is one of the most studied model organisms in biology. Flies appear to use bet-hedging as an adaptive response to environmental uncertainty, exhibiting variation in their bodily development (Krams et al., 2020) and light- and temperature-preference behaviors (Kain et al., 2015). Phototactic diversity appears to be a form of fly personality and mechanistic experiments identified the neurotransmitter serotonin (5-HT) as a suppressor of phototactic variability (Kain et al., 2012). Evolutionary modeling suggested that a bet-hedging strategy underlies the observed inter-individual diversity of phototactic and thermotactic choice. Specifically, it was suggested that flies from areas with large seasonal weather fluctuations should have greater behavioral unpredictability than fruit flies from warmer and more predictable climates (Kain et al., 2015). However, these predictions have never been tested using flies from different climate zones. In this study, we tested whether flies from northern Europe, adapted to a temperate climate with strong fluctuations, exhibit less predictable phototactic biases than those from southern Europe and tropical Africa. To assess whether potential variation in bet-hedging between these populations depends on serotonin, we tested the phototactic behavior of fruit flies collected in Finland and Kenya with and without treating them with tryptophan (5-HTP, a precursor of 5-HT synthesis) and α-methyl-tryptophan (αMW, a serotonin-synthesis inhibitor), pharmacological agents with well-established effects on fruit fly behavior and serotonin levels (Dasari et al., 2007; Dierick and Greenspan, 2007; Neckameyer, 2010; Majeed et al., 2016; Ries et al., 2017; Hu et al., 2020).

The northernmost record of overwintering populations of *D. melanogaster* is from southwest Finland (Keller, 2007), while this cosmopolitan species (Markow, 2015) likely originated in the tropical region including Kenya (David and Capy, 1988; Lachaise et al., 1988; Baudry et al., 2004; Keller, 2007). We predicted that flies from the northern climate would be less predictable in their phototactic biases because they have adapted to a rapidly fluctuating environment with a bet-hedging strategy. This could be achieved by a reduction of 5-HT in their brains, in which case feeding these flies 5-HTP should decrease the variability of their phototactic biases. Conversely, the brains of Kenyan flies might contain more serotonin, in which case adding αMW should increase the unpredictability of their phototactic biases. Variability of phototactic biases was assessed by measuring the variability beyond expectation (VBE), which compares the observed variation to the variation expected due to sampling error (Kain et al., 2015). As the VBE is a nonparametric parameter and sensitive to outliers, it has a low signal-to-noise ratio. We have overcome this disadvantage using large sample sizes in each experimental group and nonparametric analyses.

MATERIALS AND METHODS

Insects

We studied the phototactic behavior of fruit flies sampled from wild populations near Mombasa (Kenya) and between Turku and Tampere (Finland). Local wild flies were caught using banana and yeast baited traps or collecting the pupae from compost piles in April 2017, in Kenya and in August 2017 in Finland. In either country, we collected the flies from five locations separated by a 5-km distance at least.

For behavior experiments, parental flies were kept in vials for 24-h to oviposit. When their progeny enclosed, they were removed in the first 5–7 h post-eclosion, anesthetized, and separated by sex. In this study, we tested flies grown on Formula 4–24 instant *Drosophila* media (item #173202, Carolina Biological Supply Company, Burlington, NC, USA).

Phototaxis Equipment

We studied the variability of phototaxis behavior in F1 flies. The FlyVac apparatus allowed us to measure the startled phototaxis behavior of many individual fruit flies simultaneously (Kain et al., 2012). The operational details of FlyVac are detailed elsewhere (Kain et al., 2012). In brief, FlyVac is an instrument for the rapid quantification of phototaxis behavior. Up to 32 individual fruit flies were loaded into separate phototaxis modules, each consisting of a phototactic T-maze in which the fly could choose between a light [an illuminated light-emitting diode (LED)] and dark stimulus (a non-illuminated LED). Both branches of the T-maze are equipped with an LED but only one LED is illuminated, at random, in each trial.

To begin a phototaxis session, individual flies are aspirated from its culture vial into the vertical start tube of the T-maze. After insertion, a fly climbs upward through the vertical tube of the T-maze under negative geotaxis until it reaches the choice point of the T-maze. Upon making a choice by entering one of the corridors of the T-maze, the fly is detected by an optical interrupter. This triggers recording the direction of the choice done with respect to the direction of the illuminated stimulus LED and opens a vacuum to pull the fly back into the start tube. In each trial, one LED out of two is lit at random. After completing 40 trials, the phototaxis module is deactivated and the flies are simply contained until removal. In the event that a fly does not complete 40 trials within several hours, that fly is removed from the module and further analyses. Before the trials, we have checkered whether the FlyVac apparatus itself was not affecting behavior. We have performed a long series of assays with two LEDs on and with two LEDs off. In both cases, the resulting distributions are statistically indistinguishable from the random binomial distribution.

Groups and Drug Treatments

We had three experimental groups per geographic location: flies (males and females) grown without any drugs, flies grown on food supplemented with 5-HTP and flies grown on food supplemented with αMW (Dasari et al., 2007; Dierick and Greenspan, 2007; Neckameyer, 2010; Majeed et al., 2016; Ries et al., 2017; Hu et al., 2020). Drugs were dissolved in Formula 4–24 instant *Drosophila* media. For the drug-feeding, F0 flies laid eggs in drug-containing media. Upon eclosion, adult F1 flies were assayed on days 2–3. The drug stock solutions were vortex-mixed and added to food powder. The final concentration of 5-HTP was 50 mM and the final concentration of αMW was 20 mM (Huber and Ronchetti, 2009; Kain et al., 2012).

Statistics

We first fitted the Poisson (with log link) generalized linear model (GLM) using positive light-choice count as a dependent variable and geographic location (Finland, Kenya), treatment groups (control, 5-HTP, αMW) and sex as predictors with interaction (sex × treatment groups). After determining that sex was not a significant factor, we excluded it and fitted the final GLM using geographic location, group and an interaction between geographic location and treatment group. We assessed the variation of phototactic choices by calculating the variability beyond expectation (VBE; Kain et al., 2015). This metric is equal to log2(μADobs/μADexp), where μADobs indicates the mean absolute deviation of the data from their median and μADexp is the equivalent value calculated from the binomial distribution corresponding to the sampling error expected if all individuals made light choices with identical probabilities (Huber and Ronchetti, 2009). Standard errors for VBE were calculated by bootstrap resampling (5,000 replicates) of individual flies. Pairwise comparisons of light-choice probability (LCP) between groups were made using Mann–Whitney *U* tests.

RESULTS

Light-Choice Probability (LCP)

We found that female and male flies did not differ significantly in their LCP (fraction of choices toward the illuminated LED) either in Finland or Kenya (Poisson GLM with log link, Wald $Chisq_1 = 0.66$, $P > 0.4$) and, therefore, we pooled sexes in the further analyses of LCP. We found that geographic location was a significant predictor of light-choice probability (Poisson GLM with log link, Wald $Chisq_1 = 290.8$, $P < 0.0001$). Flies were photopositive both in Kenya and Finland, choosing the light 80% and 68% of the time (**Table 1**; **Figure 1**), respectively. Kenyan flies were found to be significantly more photopositive than Finnish flies ($P < 0.001$; **Table 1**; **Figure 1**). While the main effect of treatment group on light-choice was not significant (Poisson GLM with log link, Wald $Chisq_2 = 0.449$, $P = 0.8$), however, there was significant interaction between geographic location and treatment group (Poisson GLM with log link, Wald $Chisq_2 = 40.93$, $P < 0.0001$). Feeding Kenyan flies, αMW (a serotonin synthesis inhibitor) increased their LCP significantly while feeding 5-hydroxytryptophan, a serotonin precursor (5-HTP) reduced LCP (**Table 1**; **Figure 1**). In contrast, feeding Finnish flies αMW reduced LCP, while 5-HTP increased their LCP. However, LCP of Finnish flies fed 5-HTP was still lower than the LCP of Kenyan flies (**Table 1**; **Figure 1**).

Variability Beyond Expectation (VBE)

Female and male flies had similar among-individual phototactic variability, as measured by VBE, in both Finland and Kenya (all bootstrapped distributions, $P > 0.05$) and sexes were pooled in the further analyses of VBE. Finnish fruit flies had significantly higher VBE than flies in Kenya ($P < 0.001$; **Table 1**; **Figure 2**). Feeding αMW did not affect the VBE of Finnish flies, whereas adding 5-HTP to their food significantly suppressed VBE ($P = 0.023$). In Kenyan flies, feeding αMW significantly increased VBE, while 5-HTP did not affect their VBE (**Table 1**;

TABLE 1 | Descriptive statistics of light-choice probability (LCP) and variability beyond expectation (VBE); ±SE were based on bootstrap resampling (5,000 replicates).

Geographic location	Treatment	*n*	Light choice probability (LCP) ± SE	Variability beyond expectation (VBE) ± SE
Finland	5-HTP	286	0.695 ± 0.00665	0.5278 ± 0.06943
Finland	Control	255	0.6798 ± 0.00779	0.7225 ± 0.0703
Finland	αMW	201	0.6454 ± 0.00977	0.8394 ± 0.07307
Kenya	5-HTP	175	0.7673 ± 0.00745	0.5458 ± 0.085
Kenya	Control	256	0.7964 ± 0.00564	0.3839 ± 0.07685
Kenya	αMW	197	0.8379 ± 0.00663	0.6392 ± 0.07468

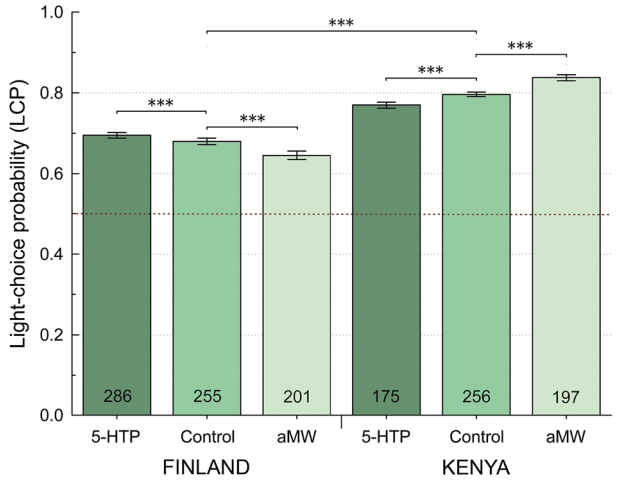

FIGURE 1 | Light-choice probability (LCP) by site and pharmacological treatment. 5-HTP, Tryptophan, a precursor of serotonin synthesis; αMW, α-methyl-tryptophan, a serotonin-synthesis inhibitor. Error bars are ±1 SE, calculated by bootstrap resampling; ***$P < 0.001$.

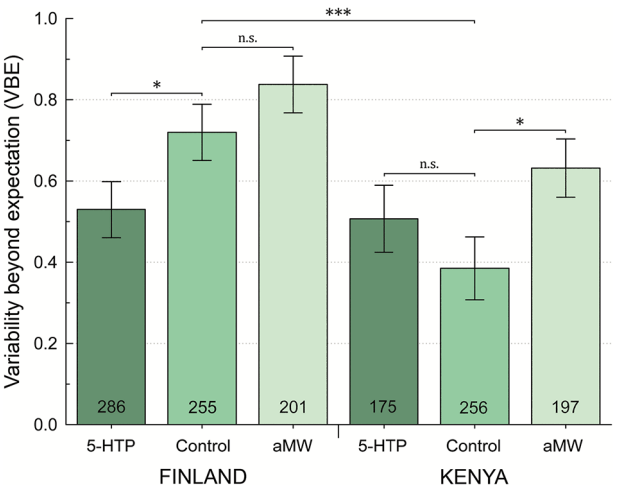

FIGURE 2 | Behavioral variability beyond statistical expectation (VBE) by site and pharmacological treatment. On a log2 scale, VBE = 0 indicates no excess variability, and VBE = 1 indicates twice as much variability as would be expected by chance alone. Error bars are ±1 SE, calculated by bootstrap resampling; $^{n.s.}P > 0.05$, *$P < 0.05$, ***$P < 0.001$ represent one-tailed significance.

Figure 2). Importantly, feeding 5-HTP made VBE of Finnish flies similar to VBE of Kenyan flies (**Table 1**; **Figure 2**).

DISCUSSION

In this study, we examined light-choice probability and the variation beyond expectation of the light choice probability in fruit flies from tropical and boreal climates. Although all flies were raised and tested in identical conditions, Kenyan fruit flies originated from areas with stable and predictable climate, while Finnish fruit flies originated in the northernmost limits of the species' distribution range, a zone characterized by an intensely fluctuating climate. We found that flies from the southern climate and were more strongly attracted to light (higher LCP), and more consistent across individuals (lower VBE) than flies from the northern climate. Conversely, flies from the higher latitude site were less attracted to light and less consistent across individuals. Bet-hedging theory predicts that higher phenotypic diversity may be adaptive under conditions of less predictable climate. Kain et al. (2015) developed a computational model suggesting that this hypothesis is plausible specifically with respect to *Drosophila* light-choice behavior. The results of this study provide empirical evidence in support of this model using fly strains caught at geographic sites with differential climatic variability (Akhund-Zade et al., 2020).

Previous work shows that genetic variation within lab strains likely cannot account for the variation of phototactic responses (Kain et al., 2012). In contrast, VBE varied significantly between flies collected in eastern Africa and northern Europe, suggesting that genetic factors underlie differences in the magnitudes of variation. A genetic basis for variability has been found in several other *Drosophila* behaviors including locomotor bias (Ayroles et al., 2015) and odor preference (Honegger et al., 2019). With respect to phototaxis, it was found that the gene *white* has an important role as an importer of metabolic precursors of serotonin (Kain et al., 2012). In this study, we significantly decreased VBE of Kenyan flies by feeding them αMW, an inhibitor of serotonin biosynthesis. Feeding Finnish fruit flies 5-HTP, a serotonin biosynthesis precursor, significantly reduced their VBE. Thus, manipulations to reduce serotonin levels in Kenyan flies and increase them in Finnish flies made VBE of these groups statistically similar. Notably, feeding Kenyan flies 5-HTP did not significantly reduce the VBE of these flies. Likewise, feeding Finnish flies αMW did not increase their VBE. These results suggest that a possible ceiling effect in Kenyan flies renders them insensitive to additional serotonin. Conversely a floor effect in Finnish flies may explain their insensitivity to serotonin inhibitors (Lam, 1996). However, we cannot exclude the possibility that the 5-HT-related effects on light choice and the variability of choices are also due to independent mechanisms. For example, independent genetic effects on the mean and variability of light choice were observed when the *white* gene was muted (Kain et al., 2012).

We found that Finnish flies exhibited high VBE for phototaxis, similarly to what was observed in a laboratory strain, *D. melanogaster* w^{1118} (Kain et al., 2012). These flies have white eyes (Morgan, 1910; Green, 1996) due to a mutation in the gene *white*, which is a central part of the eye-pigmentation pathway (Ferreiro et al., 2018). This gene encodes White, an ATP binding cassette transporter (Pepling and Mount, 1990), that heterodimerizes with either Brown or Scarlet proteins, encoded by brown and scarlet genes to transport guanine or 5-HTP, respectively. In neurons, these transporters contribute to the biosynthesis of amines. It has been shown that *white* mutants have significantly reduced levels of the neurotransmitters

serotonin (up to five times lower), dopamine, and histamine (Borycz et al., 2008; Sitaraman et al., 2008), especially in glia and neurons of the brain (Borycz et al., 2008). These diminished concentrations of the neurotransmitters in *white* mutants (Borycz et al., 2008; Sitaraman et al., 2008) have multiple consequences on a variety of neurological phenotypes affecting male courtship behavior (Zhang and Odenwald, 1995; Anaka et al., 2008; Lee et al., 2008), anesthesia resistance (Campbell and Nash, 2001), aggressive behavior (Hoyer et al., 2008), spatial learning and olfactory learning (Diegelmann et al., 2006; Anaka et al., 2008; Sitaraman et al., 2008), duration of periods of locomotion recovery following anoxia (Xiao and Robertson, 2016), sensitivity to ethanol (Chan et al., 2014), sensitivity to certain tactile stimuli (Titlow et al., 2014) and propensity to retinal degeneration (Ferreiro et al., 2018). Although Finnish fruit flies have normal red eyes, they displayed average light preference (68%) and VBE (0.72) similar to the values seen in *white* mutants. For example, w^{1118} mutants chose light 61% of the time and their VBE is ∼0.87, values which are closer to those in Finnish flies than, for example, flies of the standard lab wild type strain Canton-S (76% and 0.56, respectively; Kain et al., 2012).

Common factors may be responsible for the behavioral metrics of Finnish and w^{1118} flies. We found that 5-HTP significantly affected VBE of Finnish flies, while it had no effect on VBE of Kenyan flies. The same pattern was observed in w^{1118} and Canton-S flies, respectively (Kain et al., 2012). This suggests that the brains of African flies contain a higher concentration of serotonin, perhaps because their food sources are more diverse and may contain more metabolite precursors than Finnish flies. Tryptophan, a serotonin precursor, is an essential amino acid because animals cannot synthesize it but instead must obtain it through their diet. While African flies often enjoy the availability of different fruits, mushrooms, sap fluxes year-round, Finnish fruit flies have much shorter summer season in general, with reduced availability of rotting and decaying fruits and mushrooms in particular (Sardeshpande and Shackleton, 2019). The depletion of tryptophan from the diet has been used to assess brain serotonergic function in humans (Lam, 1996). This procedure is capable of rapidly lowering brain tryptophan levels in human patients by over 80% within just a few hours (Young et al., 1985), which may have immediate effects on depression patients (Smith et al., 1997; Neumeister et al., 1998) such as deviations from normal behavior and lowered food intake (Rantala et al., 2018, 2019).

Theory predicts that the relative stability of the local climate in Kenya should favor heritable and lower variability phototactic preferences, i.e., a strategy with less stochastic bet-hedging (Hopper, 1999). In such strategies, the current mean phenotype always lags environmental fluctuations, because evolution by natural selection is not instantaneous. In predictable environments, the penalty for this lag is minimized. By contrast, Finnish flies showed significantly more variable phototactic preferences, suggestive of an adaptive bet-hedging and consistent with previous modeling of bet-hedging in thermal preference behavior (Kain et al., 2015). Interestingly, adaptations for heat resistance have the potential to improve cold resistance (Condon et al., 2014). This shows that adaptations to extreme temperatures improve not only the ability to withstand a particular deviation from mean temperatures, but also the magnitude of temperature variation. Moreover, the ability to tolerate extreme temperatures is improved in populations that evolve in fluctuating environments relative to when populations are exposed to a stable increase of high temperatures (Condon et al., 2014; Tobler et al., 2015). The high VBE of Finnish flies, which in the wild may result in a variety of thermal experiences, may serve as parallel adaptation to life in relatively unpredictable thermal and visual environments, leading flies to find conspecifics, breed and oviposit in a variety of conditions, rather than wait for specific optimal conditions that might not arrive in a particular season.

In a population utilizing a bet-hedging strategy, individuals exhibiting a wide variety of preferences are born continuously across a season. If the summer is cooler, spring-adapted individuals will survive, while summer-adapted flies will survive if the summer is hot and long (Bergland et al., 2014). Kawecki (2000) has suggested that the phenotypic expression of genetic variation can be suppressed, and heritability reduced under fluctuating selection. Dynamic modulation of variability-suppressing serotonin is a potential mechanism to tune the canalization of the phototactic phenotype. To test this possibility, one could measure VBE and 5-HT concentration in the brains of flies, born during hot and cool summers near the northernmost areas of their distribution ranges. Our results suggest that plastic responses to environmental differences, which is another major strategy for dealing with environmental heterogeneity, is not a likely explanation for the observed differences between African and European flies. The flies of both populations were grown under identical conditions and we are not aware of any environmental fluctuations to which a plasticity strategy could respond.

While Kain et al. (2012) observed significant effects of 5-HTP on the VBE of flies, this treatment did not show any influence on light-choice probability in their study. However, we found significant effects of feeding 5-HTP and αMW on LCP, which depended on the origin of the strain. In addition, we observed effects on LCP of line origin, with Kenyan flies ∼10% more photopositive. In Finnish flies, feeding 5-HTP did not affect LCP, while feeding αMW significantly reduced it, which was the opposite of what we observed in the case of VBE in these flies. Feeding 5-HTP significantly lowered LCP and feeding αMW significantly raised LCP in Kenyan flies. Thus, 5-HTP decreased the light choice probability in Kenyan flies and did not affect it in Finnish flies. Importantly, Kenyan flies on control media chose the light more often than Finnish flies on control media. Kenyan flies are likely to have a higher concentration of 5-HT in the brains, at least when fed natural diets.

It is possible that differential levels of serotonin do not explain the mean LCP of these strains, since serotonin or its precursor 5-HTP have been previously reported to decrease photopositivity in larval bryozoans (Pires and Woollacott, 1997). There may also be genetic background by serotonin-exposure effects. However, dopamine was previously reported to increase light choice (Pires and Woollacott, 1997). *White* mutants have

reduced concentrations of dopamine in the brain (Borycz et al., 2008; Sitaraman et al., 2008) and if the neuromodulatory state of Finnish flies mirrors that of *white* mutants, they may also have lower dopamine levels. This in turn might explain their lower LCP, while lower serotonin could explain their higher VBE. Kain et al. (2012) did not find any effect of dopamine drugs on VBE or LCP of different strains of fruit flies including *white* mutants. However, it has been shown that dopamine affects the production and release of melatonin (González et al., 2012), a key driver of biological rhythm (Arendt and Skene, 2005). Melatonin production might be disrupted in the brains of Finnish flies to ensure activity during long summer days at high latitudes indicating that dopamine of boreal fruit flies, especially the receptor subtypes and the density of receptors deserve a special attention in future research.

Importantly, 5-HT is a precursor of melatonin (Richter et al., 2002), and 5-HT is also regarded as a substance affecting physiological rhythms according to the light–dark cycle in invertebrates (Hardeland and Poeggeler, 2003). Kenyan and Finnish flies likely have different diurnal rhythms and sleep patterns: While there is a relatively regular day/night cycle in the tropical zone, Finnish flies enjoy never-ending daylight for up to two months at high latitudes. This may affect their serotonergic neural regulation because melatonin may be in low demand and not metabolized much during the northern summer, perhaps allowing the accumulation of elevated 5-HT in neural tissues in summer. Besides a leading role of melatonin in the determination of sleep/wake cycles, it is also a potent antioxidant with a proposed role in immune function in invertebrates (Tan et al., 2009). The suppression of nocturnal production of melatonin has detrimental effects on antioxidant systems of organisms (Jones et al., 2015) which may facilitate the bet-hedging strategy of invasive species at high latitudes.

Flies that follow a bet-hedging strategy might only attain environmental conditions well-matched to their behavioral biases if they live through long periods of poorly matched conditions. Thus, there is likely an interplay between generation/lifespan length and the timescale of environmental fluctuations. Indeed, modeling suggests that bet-hedging is an adaptive response to environmental fluctuations at specific timescales roughly corresponding to the lifespan (Krams et al., 2020). Melatonin is an antioxidant, and may lengthen the lifespan of flies (Terán et al., 2012). Thus, it has the potential to affect evolutionary behavioral strategies both directly through the neuromodulatory state, but also indirectly through an effect on lifespan. These hypotheses call for precise measurements of 5-HT, melatonin, dopamine and behavior in fruit flies across the season and the south-north gradient of their distribution range.

CONCLUSIONS

Proving that phenotypic variability reflects a bet-hedging strategy is a tall order. A formal approach to this question (Simons, 2011), stipulates six increasingly convincing categories of evidence for bet-hedging: (1) description of a potential bet-hedging trait, (2) measured variability of the environmental characteristics pertinent to the bet-hedging strategy, (3) genotype-specificity of variability or a genetic mechanism underlying it, (4) identified fitness consequences of phenotypic variations, (5) experiments showing that bet-hedging provides a geometric-mean fitness advantage in a fluctuating environment, and (6) an empirical match to the predicted optimal magnitude of phenotypic fluctuation in response to environmental fluctuation. With respect to phototaxis in flies, this work provides evidence in the first three categories. Flies vary in their phototactic preferences, and this may reflect a bet-hedging strategy in response to fluctuations in luminance and thermal fluctuations, which are greater in Finland than in Kenya. Fly strains from these sites exhibit lab-stable differences in variability, consistent with a genotypic basis to bet-hedging (Category 3). We speculate that individual phototactic preferences may have fitness consequences (Category 4) through temperature-dependent effects on life history (Kain et al., 2015; Akhund-Zade et al., 2020) or differential access to environmental resources. However, establishing this link empirically and acquiring evidence for bet-hedging in Categories 5 and 6 will require additional studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

IK, TK, RK and BB conceived and designed the study. TK, RK, IK, GT, SP, PJ, MM and MR performed the study, collected and extracted data. GT, DE, RK and IK analyzed the data. JM, SP and IK built the equipment. BB, TK, MR, PJ, SP, JM and MM participated in data analyses, results interpretation, and drafting the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by Fulbright Program of the US Department of State. Funding was provided by the Latvian Council of Science (Latvijas Zinātnes Padome; grants lzp-2018/1-0393, lzp-2018/2-00057, and lzp-2020/2-0271), the Estonian Research Council (Eesti Teadusagentuur; grant PUT1223). BB was supported by a Sloan Research Fellowship, a Klingenstein-Simons Fellowship Award, a Smith Family Odyssey Award, a Harvard/MIT Basic Neuroscience Grant, and National Science Foundation grant no. IOS-1557913.

ACKNOWLEDGMENTS

We thank Prof. Christine R.B. Boake, Prof. Todd M. Freeberg and Prof. Gordon M. Burghardt for their support during initial phases of this study. Professors Jae H. Park, Mariano Labrador, Ranjan Ganguly and Joshua N. Bembenek kindly provided access to their lab facilities at Knoxville. We also thank Raimonds Subotjalo and Kristers-Raivo Krams for their support at various stages of the study.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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II PUBLICATION

II PUBLIKĀCIJA

frontiers | Frontiers in Behavioral Neuroscience

TYPE Original Research
PUBLISHED 25 May 2023
DOI 10.3389/fnbeh.2023.1189301

Check for updates

OPEN ACCESS

EDITED BY
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RECEIVED 18 March 2023
ACCEPTED 09 May 2023
PUBLISHED 25 May 2023

CITATION
Krama T, Munkevics M, Krams R, Grigorjeva T, Trakimas G, Jõers P, Popovs S, Zants K, Elferts D, Rantala MJ, Sledevskis E, Contreras-Garduño J, de Bivort BL and Krams IA (2023) Development under predation risk increases serotonin-signaling, variability of turning behavior and survival in adult fruit flies *Drosophila melanogaster*. *Front. Behav. Neurosci.* 17:1189301. doi: 10.3389/fnbeh.2023.1189301

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Development under predation risk increases serotonin-signaling, variability of turning behavior and survival in adult fruit flies *Drosophila melanogaster*

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The development of high-throughput behavioral assays, where numerous individual animals can be analyzed in various experimental conditions, has facilitated the study of animal personality. Previous research showed that isogenic *Drosophila melanogaster* flies exhibit striking individual non-heritable locomotor handedness. The variability of this trait, i.e., the predictability of left-right turn biases, varies across genotypes and under the influence of neural activity in specific circuits. This suggests that the brain can dynamically regulate the extent of animal personality. It has been recently shown that predators can induce changes in prey phenotypes via lethal or non-lethal effects affecting the serotonergic signaling system. In this study, we tested whether fruit flies grown with predators exhibit higher variability/lower predictability in their turning behavior and higher survival than those grown with no predators in their environment. We confirmed these predictions and found that both effects were blocked when flies were fed an inhibitor (αMW) of serotonin synthesis. The results of this study demonstrate a negative association between the unpredictability of turning behavior of fruit flies and the hunting success of their predators.

We also show that the neurotransmitter serotonin controls predator-induced changes in the turning variability of fruit flies, regulating the dynamic control of behavioral predictability.

KEYWORDS

***Drosophila melanogaster*, behavioral predictability, serotonin, survival under predation, turning behavior**

Introduction

Living organisms adapt to varying environmental conditions by attempting to modify their morphological, biochemical, and behavioral phenotypes (Xue et al., 2019). Predation has been shown to have profound lethal and non-lethal (Lima, 1998) impacts on prey individuals, affecting their behavior (Hulthén et al., 2021), phenotype development (Krams et al., 2020), fitness (Zanette and Clinchy, 2020; Allen et al., 2022), population structure and evolution (Yartsev, 2017; Dudeck et al., 2018). Prey individuals respond to predator acoustic, visual, chemical, and other cues, which improve the chances of prey to escape predator attacks (Lima, 1998; Preisser et al., 2005; Peckarsky et al., 2008; Voelkl et al., 2016; Zanette et al., 2019; Krams R. et al., 2021). When developing under predation risk, prey individuals often grow smaller, more agile, less palatable, or more cryptic, conferring fitness benefits associated with a modified phenotype (Krams et al., 2016). Fruit flies (*Drosophila melanogaster*) raised during the larval stage together with jumping spiders had more nitrogen in their bodies and lower body lipid reserves, while they had a higher climbing speed in the negative geotaxis test than flies grown without spiders (Krams et al., 2016). Moreover, fruit flies grown together with predators had significantly higher adult survival ability when exposed to predation than flies grown in a predator-free environment (Krams et al., 2016). This shows that predator exposure in ontogeny may directly affect survival in adulthood. However, it is not always clear what changes in the neural and behavioral phenotypes facilitate the escape performance of fruit flies at risk of predation.

Locomotor activity has been shown to change adaptively during the evolutionary "arms race" between prey and predator by enhancing the predator escape ability of prey individuals (Moore and Biewener, 2015; Moore et al., 2017). Jumping spiders are ambush predators whose attacking repertoire involves direct attacks triggered by the approaching prey (Rößler et al., 2022). Ambush predators remain concealed and motionless until the prey comes within ambush distance before pouncing. If the prey survives in the initial attack, the predator often does not pursue it (Scharf et al., 2006). Although ambush predators are not supposed to actively rely on predictions of the prey's behavior (Caraco and Gillespie, 1986; Mischiati et al., 2015; Moore and Biewener, 2015), the lower predictability of approach trajectories of prey may affect the chances of the prey to approach the predator's ambush distance. Moreover, so-called "protean" behavior is defined as a sufficiently unpredictable response to prevent a predator from anticipating its prey's future position or actions (Humphries and Driver, 1970; Richardson et al., 2018). However, the exact predictability of potentially "protean" prey behaviors has received limited observational and experimental attention.

Fruit flies exhibit striking locomotor handedness during their exploratory behavior (Buchanan et al., 2015; de Bivort et al., 2022), one example of the preferential performance of a behavior on one side of the body. During exploratory walking in symmetrical environments, individual fruit flies exhibit significant bias in their left vs. right/counter-clockwise vs. clockwise locomotor choices, with some flies being strongly left-biased or right-biased. This behavioral idiosyncrasy is present across different fly lines and genotypes. Moreover, the flies differing in neural state (Buchanan et al., 2015) or genotype (Ayroles et al., 2015) differed in the extent of left vs. right turning bias. Specifically, the magnitude of turning bias variation is under the control of columnar neurons within the central complex, a brain region implicated in motor planning and execution of fruit fly behavior (Buchanan et al., 2015). Turn bias variability has a complex genetic architecture involving many genes, particularly those involved in circuit development during pupation, including specifically *teneurin-A* (Ayroles et al., 2015) that encodes a protein involved in synaptic partner matching (Mosca, 2015). Silencing the central complex columnar neurons or knocking down *teneurin-A* expression increased exploratory laterality in fruit fly turning behavior, with more extreme leftiness and rightiness, decreasing the predictability of turning choices across individuals. In the mathematical limit, a population with maximal turn bias unpredictability across individuals (composed of equal parts extreme righties and extreme lefties) would consist of animals with high within-individual predictability (making exclusively right or left turns, respectively). But in experiments, examining microhabitat occupancy, a positive correlation was observed between population-level behavioral predictability and individual predictability (Stamps et al., 2013). In this study, we use "predictability" to refer to variability in behavior at the population level, across individuals.

Neurotransmitters are known to control the predictability of behavior (Maloney, 2021). The predictability of phototaxis in flies is under the control of the neurotransmitter serotonin (5-HT), and the lowest predictability of turning choices were found in white-eyed w^{1118} mutants (Kain et al., 2012; Krams I. A. et al., 2021). White-eyed flies have 32% less 5-HT in their heads than the brains of red-eyed fruit flies (Borycz et al., 2008; Krstic et al., 2013). 5-HT also regulates the predictability of odor preferences in flies (Honegger et al., 2020) and locomotor activity in the roundworm *C. elegans* (Stern et al., 2017; Nasser et al., 2022). With respect to turn bias variability, both increasing and decreasing 5-HT with metabolic drugs had small effects of reducing turn bias variability, averaged across many genotypes (de Bivort et al., 2022).

Applying serotonin precursor increases variability in locomotor speed, and there is a bidirectional effect of altering serotonin levels on variability in higher-order left-right turn sequences (de Bivort et al., 2022). All these effects are small, but they generally suggest a role for serotonin in decreasing locomotor predictability. It has been recently shown that predator-induced stress influences a number of 5-HT-associated behavioral and physiological effects in fruit flies grown together with spiders during larval development (Krama et al., revision 2, personal observation). This implies that predators may influence the brain to dynamically regulate the predictability of the turning behavior of fruit flies to improve their survival under predation risk.

In this study, we tested whether fruit flies reared with spiders exhibit lower predictability in their turning behavior in Y-mazes (Buchanan et al., 2015), compared to flies reared in predator-free environments. We also studied the survival of fruit flies grown with and without spiders. To investigate the role of 5-HT in regulating antipredator behavior, we fed fruit flies raised with spiders and flies raised without spiders 5-HTP (a precursor of 5-HT) and αMW (a serotonin-synthesis inhibitor). We hypothesized that predator presence during larval development might make the turning behavior of adult fruit flies less predictable and improve their survival. We predicted that feeding αMW might make turning choices of flies reared together with predators more predictable (de Bivort et al., 2022) and decrease their survival. We studied male fruit flies only because a large portion of the body of a mated/unmated female is composed of developing eggs and reproduction-related tissue, which may influence body mass, body size, metabolism, and antipredator behavior, potentially affecting predator preferences (Burggren, 2017). Individual-to-individual differences in experimental behavioral observations reflect persistent idiosyncrasies requiring large samples (Sih et al., 2004; Mollá-Albaladejo and Sánchez-Alcañiz, 2021). Small mazes arrayed in parallel allow the measurement of behavior of hundreds of individual flies simultaneously (de Bivort et al., 2022) and high-powered inference of the effects of experimental manipulations (Brown and de Bivort, 2018).

Materials and methods

Prey, predators, developmental speed, and the main treatment groups

The wild type strain of *D. melanogaster* [Oregon-R-modENCODE (#25211)] was obtained from Bloomington Drosophila Stock Center (IN, US). This line of OR was inbred for 10 generations before behavioral experiments were collected. We reared our stock flies at the University of Tennessee-Knoxville at 24 ± 1°C, at 40% relative humidity with a 12/12 h light/dark cycle.

We used adult jumping spiders (*Phidippus apacheanus*) as predators to affect the development and behavior of fruit flies. *P. apacheanus* is widely distributed across the US, and the spiders are easy to maintain in the lab because they readily consume both larvae and adults of *D. melanogaster* (Krams et al., 2016). The adult spiders were caught in Florida and delivered by the supplier phids.net.

Developmental speed significantly affects body mass, elemental body composition, food uptake, and fat metabolism of *D. melanogaster* (Krams et al., 2020). This makes the flies with rapid, intermediate, and slow development different in their biochemical and morphological phenotype, which needs to be considered when planning research. To avoid the developmental speed-related confounding effects, we used only rapidly developing fruit flies in this study. We defined rapidly developing flies as individuals that eclose between 9.5 and 10.0 days after egg-laying (Krams et al., 2020). Rapidly growing fruit flies experience relatively low stress levels during ontogeny (Krams et al., 2020).

We isolated fruit flies using carbon dioxide anesthesia within 6–7 h after eclosion. Ten F0 females and ten males were placed for 24 h in one vial (Flystuff polystyrene vials; Genesee Scientific, El Cajon, CA, USA, 24 mm inner diameter × 95 mm height) containing 6 ml of Cal Tech medium. After 24 h, the adults were removed, and the vials were placed horizontally on the floor of Plexiglas jars (10 cm height × 12 cm diameter). The density of F1 first-instar larvae across the vials was similar, and we averaged the density to 120 larvae/vial by removing extra larvae with a squirrel brush (Krams et al., 2016). Vials with *Drosophila* larvae were randomly divided into two groups: one that was exposed to spiders and one that was not. In the spider-treated group, a single *P. apacheanus* individual was also included in each Plexiglas jar. The vials did not have stoppers, giving the spider free access to the developing flies (as well as the fly media). Developing flies were also exposed to the odor of the spider throughout the container. Flies for behavioral and survival assays were removed the day after they eclosed, without anesthesia, and transferred to drug-treated vials as described below.

Neurotransmitter treatments

We had two main experimental groups of *D. melanogaster*: flies grown together with predators and flies grown with no predators (**Figure 1A**); each of these two groups was further divided into three subgroups: flies raised on food supplemented with 5-HTP, flies grown on food supplemented with αMW, and flies grown without any drugs (Neckameyer, 1996; Dasari et al., 2007; Dierick and Greenspan, 2007; Majeed et al., 2016; Ries et al., 2017; Hu et al., 2020; Krams I. A. et al., 2021; **Figure 1A**). The drug stock solutions were vortex-mixed and added to food powder. 5-HTP and αMW were dissolved in Cal Tech instant media (United States Biological, Salem, MA, USA). The final concentration of 5-HTP was 50 mM, and the final concentration of αMW was 20 mM (Kain et al., 2012; Krams I. A. et al., 2021). The flies were 5–7 days old at the moment of behavioral experiments. Dierick and Greenspan (2007), by using HPLC, showed that 5-HTP feeding significantly increases the brain 5-HT within 3 days of treatment, while αMW significantly decreases the amount of brain 5-HT during 4 days of treatment. Honegger et al. (2020) confirmed similar effects (∼8× reduction of 5-HT with αMW treatment; ∼20× increase with 5-HTP) using ELISA assays.

Turning behavior

Since using variance as a phenotypic trait requires large sample sizes (Caballero et al., 2021), we used a high-throughput assay

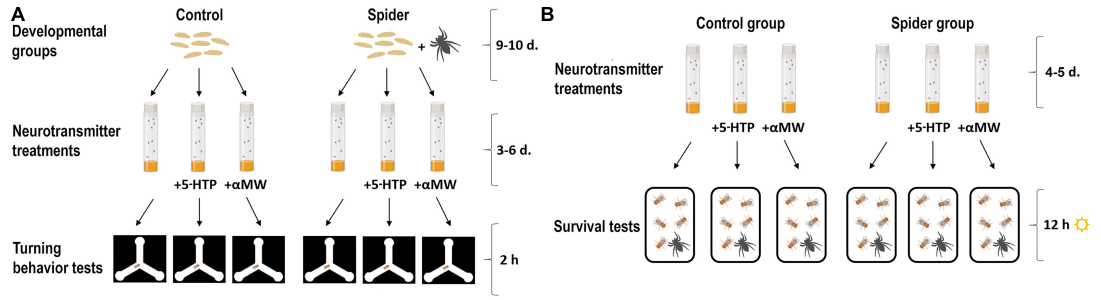

FIGURE 1
Schematic of the turning assay **(A)** and the survival experiment **(B)** (this figure was made using some sample images from BioRender.com).

to monitor the behavior of individual flies placed into individual Y-mazes (Ayroles et al., 2015; Buchanan et al., 2015). We put flies into an array containing 95 individual Y-mazes consisting of three symmetrical arms (each 12 mm long) fabricated from laser-cut acrylic (**Figure 1A**). Maze arrays were illuminated from below with a grid of 100 white LEDs (5500K, Knema) below acrylic diffusers. Maze arrays were imaged with 2MP digital cameras (Point Gray), and the X-Y positions of each fly's centroid were automatically tracked and recorded with software custom written in LabView (National Instruments, USA) (Buchanan et al., 2015). We recorded the turning behavior of 3–6-day old flies, the standard age for measuring this behavior, for 2 h. Data from the small portion of individuals making fewer than 30 turns were discarded. Each fly was used only once.

To quantify turning predictability (the variability in turning bias across individuals), we computed the MAD, the median of the absolute deviation from each observation's median (Buchanan et al., 2015), a metric of variability that is robust to outliers. We estimated MAD for each experimental group.

Survival tests

We tested six experimental groups (2 spider conditions × 3 drug conditions): (1) fruit fly males grown without *P. apacheanus* spiders and without any drugs, (2) male flies grown without spiders on food supplemented with 5-HTP, (3) male flies grown without spiders on food supplemented with αMW, (4) male flies grown together with spiders on food without any drugs supplemented, (5) males raised with spiders on food supplemented with 5-HTP, (6) males grown together with spiders on food supplemented with αMW (**Figure 1B**). Upon eclosion, adult F1 flies were assayed on days 4–5.

To measure survival, we used ten Plexiglas jars (10 cm height × 12 cm diameter) and placed ten fruit flies of an experimental group into each jar (**Figure 1B**). Thus, we had ten jars for each survival group (48 jars containing 480 fruit flies) for 12 h during daylight time (Krams et al., 2016). We did not use carbon dioxide anesthesia to move fruit flies from their stock vials to survival jars. During survival tests, we placed one young (c. 6–7 months old) *P. apacheanus* spider and one vial containing fruit fly food into each plastic jar (**Figure 1B**). The spiders had access to water in a polycarbonate dish and a fitted luffa sponge. The spiders were deprived of food for c. 10 h before survival tests. Each spider was used only once.

Statistics

To compare behavioral MADs across experimental groups, we used the permutation test. The data table of the proportion of right turns taken was shuffled, and the obtained MAD scores among randomized groups were compared to those of unshuffled data. The procedure was repeated 99,999 times, and *P*-values were calculated as the proportion of instances when the shuffled difference between group pairs was larger than the unshuffled difference. We performed a two-way ANOVA to assess the effect of development conditions and drugs added to the food on the subsequent survival of adult flies under predation risk. Tukey's honest significance test followed the analysis. Turn bias (proportion of right turns) was compared between groups using Kruskal–Wallis Test by ranks. One Sample Wilcoxon Signed Rank Test was used to assess turn bias deviation from equal amount of right and left turns. We also compared the number of turns taken by fruit flies in the y-maze per minute using the Mann–Whitney *U* test.

Data analyses were performed in the R environment (version 4.1.0) (R Core Team, 2021). *P*-values of multiple comparisons were adjusted using Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995). All differences were considered statistically significant when $P < 0.05$.

Results

Variability of turning behavior

Turn bias variability of male fruit flies grown with spiders (MAD = 0.11, $N = 153$ flies) was significantly higher than that of control flies (MAD = 0.08, $N = 143$) grown in a predator-free environment (Permutation test: $P = 0.006$; **Figure 2**). Feeding 5-HT to flies reared with spiders (MAD = 0.12, $N = 116$) did not increase the turning variability ($P = 0.34$) while feeding these flies αMW (MAD = 0.10, $N = 140$) significantly decreased turn bias variability ($P = 0.021$; **Figure 1**). Feeding 5-HTP ($P = 0.33$) and αMW ($P = 0.12$) did not affect the variability of turning behavior of control fruit flies (**Figure 2**).

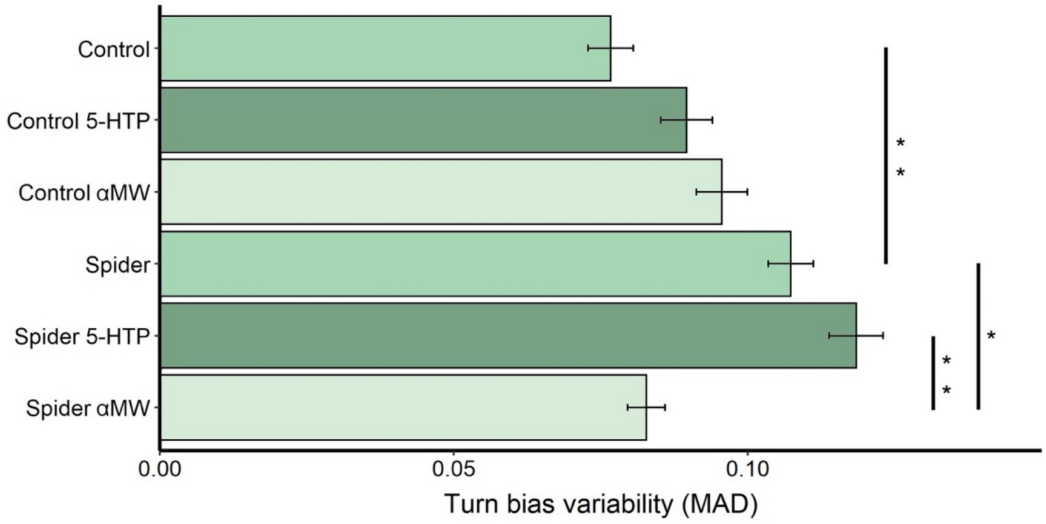

FIGURE 2
Turn bias variability (MAD) of fruit flies reared with and without spiders receiving different drug treatments. Error bars are ± SE estimated by bootstrap resampling. Asterisks indicate significant differences according to permutation tests: *0.05 > P > 0.01; **0.01 > P > 0.001.

Handedness and the number of turns in the y-maze

The proportion of the right turns (turn bias) did not differ among the groups of flies (Kruskal–Wallis: $\chi 2 = 6.41$, $P = 0.268$; Figure 3). Proportion of right turns by each group was also not significantly different from 0.5 (Wilcoxon tests: all $Ps > 0.05$; Figure 3), i.e., an equal number of right and left turns in each group.

Flies reared with spiders made significantly fewer turns per unit time (2.6 ± SD 1.3 turns/min) in the Y-maze compared to control flies (3.4 ± 1.5 turns/minute) (Mann–Whitney test: $P = 0.0001$; Figure 4). Feeding 5-HTP to flies reared with spiders significantly increased the turn rate (3.4 ± 1.4 turns/min) ($P < 0.0001$), whereas feeding them αMW had no significant effect (2.7 ± 1.3 turns/min) ($P = 0.50$; Figure 4). Feeding αMW to control flies significantly decreased the turn rate (2.6 ± 1.51 turns/min) ($P = 0.0003$), whereas feeding them 5-HTP had no significant effect (3.5 ± 1.71 turns/min) ($P = 0.94$; Figure 4).

Survival

When exposing adult flies to predation for 12 h, their survival was significantly affected by predator presence during the larval development (two-way ANOVA: $F_{1,54} = 81.37$, $P < 0.0001$), drug treatment ($F_{2,54} = 14.76$, $P < 0.0001$), and an interaction of both those factors ($F_{2,54} = 12.57$, $P < 0.0001$). Significantly more flies survived if they were reared under predator presence (mean survival: 62% ± SD 11.4%, $N = 10$) compared to the control group (30 ± 9.4%, $N = 10$) (Tukey HSD: $P < 0.0001$; Figure 5). Feeding flies reared with predators 5-HTP did not significantly affect their survival (65 ± 8.5%, $N = 10$) ($P = 0.985$; Figure 5), while feeding αMW significantly decreased their survival (35 ± 7.1%) ($P < 0.0001$; Figure 5). Feeding 5-HTP (32 ± 6.3%, $N = 10$) ($P = 0.998$; Figure 5) or αMW (30 ± 15%, $N = 10$) ($P = 1.00$; Figure 5) did not significantly affect the survival of flies of the control group.

Discussion

The presence of predators is known to alter prey morphology (McCollum and Leimberger, 1997; Hossie et al., 2010) and exert selective pressure on prey escape ability (O'Steen et al., 2002; Krams et al., 2016; Janssens and Stoks, 2018). In this study, we found that the turning choices of fruit flies grown with predators are less predictable than that of flies grown in a predator-free environment. We also show that flies raised with predators survived under predation by spiders significantly better than flies grown without predators. Our results suggest that the higher variability/lower predictability of turning behavior of flies grown with predators may make them better at evading predation. We also show with pharmacological experiments that the effects of predator-rearing on turning variability and survival of *D. melanogaster* are regulated by the neurotransmitter serotonin, which also regulates the variability of turning behavior (de Bivort et al., 2022). However, these serotonin-associated effects applied only for fruit flies grown with spiders.

Unpredictable and erratic turning behavior in some animals makes them more challenging to attack (Yager et al., 1990; Bilecenoğlu, 2005; Eifler and Eifler, 2014), as is seen in both experimental (Jones et al., 2011) and modeling (Richardson et al., 2018) studies. Individual insects can exhibit substantial differences in escape behaviors, even in the absence of genetic variation (Schuett et al., 2011). Our results suggest a link between less predictable turning behavior and better survival under predation risk by jumping spiders that are sit-and-wait predators. One explanation is that growing up with predators provides prey with signals that are not generated by transient contact with predators post-development. Perhaps the effect of these signals is mediated

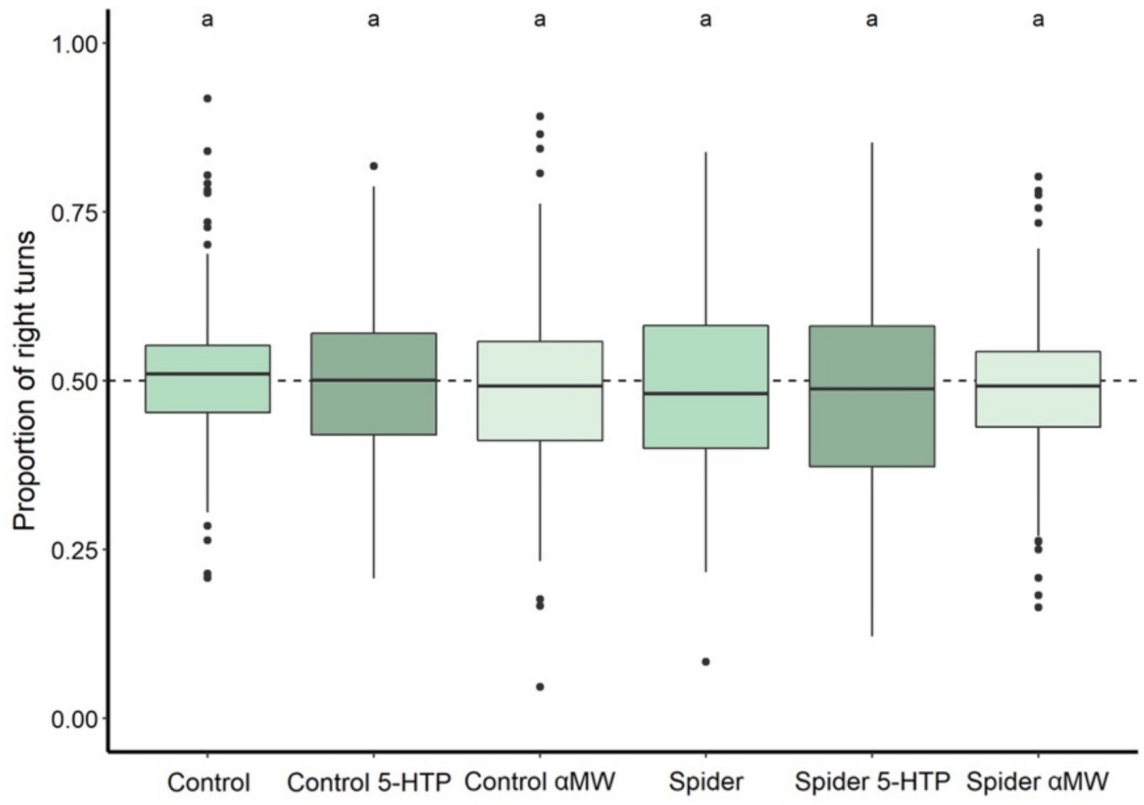

FIGURE 3
Turn bias of fruit flies reared with and without spiders and receiving different drug treatments during development. Thick lines indicate the median, and boxes indicate the 25th and 75th percentile. A dashed horizontal line indicates 0.5 proportion of right turns, a level at which flies take an equal amount of left and right turns. Thick lines indicate the median, boxes show the Q1 and Q3 quartiles, and whiskers represent the upper and lower quartile, excluding outliers. Black dots represent outliers: data points more than 1.5 times interquartile range away from Q1 and Q3. Experimental groups that are not statistically significantly different (Wilcoxon tests, $P > 0.05$) are indicated by the same letter at the top of the figure.

by serotonergic neuromodulation during prey development. This idea is consistent with the observation that flies fed αMW during development, but without predators present, showed similar adult survival in the presence of spiders as control flies, suggesting that fruit fly individuality is not solely driven by 5-HT (Maloney, 2021).

Some previous work has shown that fruit flies reared in identical lab environments show broad diversity in their phototactic choices, variability which is under the control of 5-HT (Kain et al., 2012; Krams I. A. et al., 2021). Notably, inhibiting 5-HT synthesis was associated with higher phototactic variability — here we observed that inhibiting 5-HT reduced the excess turn bias variability seen in flies reared with spiders. Geographic variation of fruit fly phototaxis was consistent with a negative relationship between 5-HT and variability of phototactic choices. Flies from northern climates grow on food relatively deficient in the metabolic precursors of serotonin and had lower predictability of phototactic choices (Krams I. A. et al., 2021). Thus, the association between 5-HT and behavioral predictability went in opposite directions in the present study and previous work examining phototaxis. These contradictory results suggest that the control of 5-HT over different behaviors may lead to different results, probably because different serotonin-responsive neuronal circuits are involved in different behaviors. To better understand the developmental, epigenetic and neurophysiological changes caused by direct predation and non-lethal predator presence, more study of behavior-specific neurobiological effects is required.

Our results support the results by Pantoja et al. (2016) examining variability in zebrafish (*Danio rerio*) antipredator locomotor behaviors. They found that zebrafish individuals show significant variation in acoustic startle responses. These responses are linked with the neurosecretion of dorsal raphe neurons (Pantoja et al., 2016). It was shown that zebrafish individuals show a higher fraction of serotonergic dorsal raphe nucleus neurons active during predator attacks. Pantoja et al. (2016) also showed that heightened 5-HT prevented habituation to predator stimuli, which improves the efficiency of antipredator behavior and survival of the prey. Together, these results suggest the importance of serotonergic signaling in the CNS and its ontogenetic development in establishing a distribution of antipredator behaviors across individuals.

The results of this study may have evolutionary implications. It is known that without phenotypic variation, there would be no evolution by natural selection. However, we show that individuals with similar genotypes raised in similar environments, except for the presence/absence of spiders, may significantly differ in their simple behavioral reactions, (such as left vs. right decision in the absence of an asymmetric stimulus in

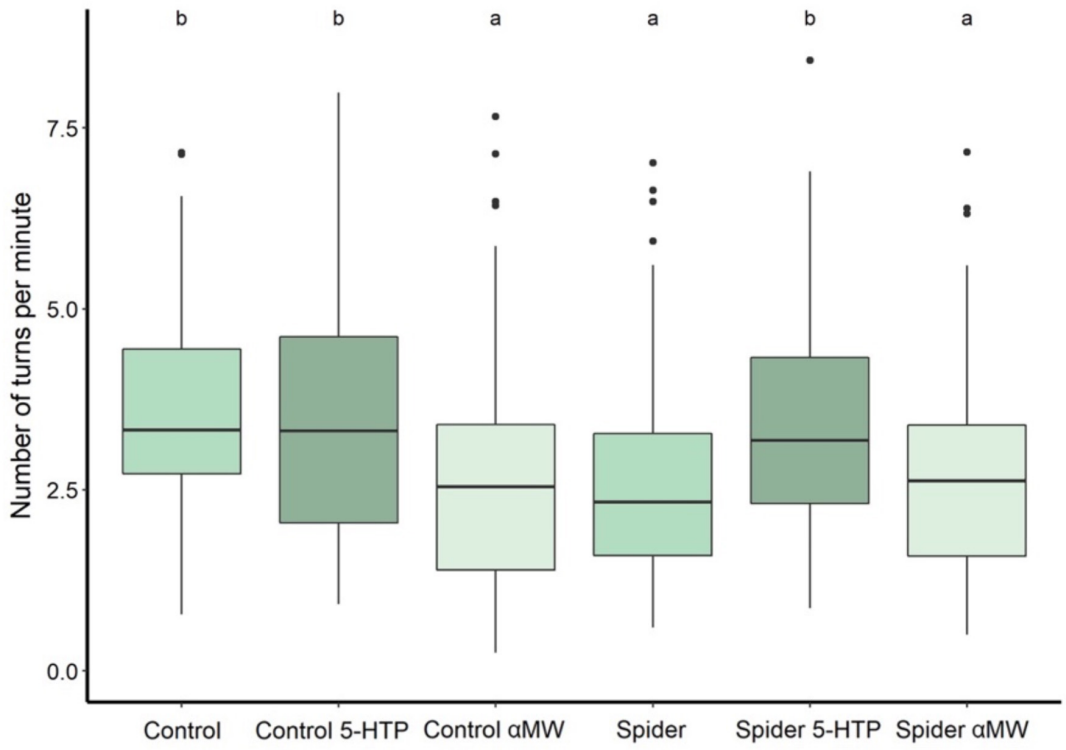

FIGURE 4
Turn rate (turns/minute) in the Y-maze of flies reared with and without spiders receiving different drug treatments. The flies reared with predators were previously exposed to predation during the larval stage, while in the control group, the flies were raised without jumping spiders. Thick lines indicate the median, boxes show the Q1 and Q3 quartiles, and whiskers represent the upper and lower quartile, excluding outliers. Black dots represent outliers (data points more than 1.5 times interquartile range away from Q1 and Q3). Experimental groups that are not statistically significantly different (Mann–Whitney tests, $P > 0.05$) are indicated by the same letter at the top of the figure.

the Y-maze). This suggests that asymmetries within the brain predispose the animal to go one way rather than the other and that neural activity influences the variation between animals (Buchanan et al., 2015). As these predispositions are relatively stable within individuals with considerable among-individual differences in behaviors (Réale et al., 2010; Buchanan et al., 2015; Roche et al., 2016; Trakimas et al., 2019), behavioral reactions of this kind are coined animal personality. Our results show that fruit flies may use a simple mechanism to dynamically regulate their behavioral individuality with individual variation in wiring and behavior as a general feature of neural circuits to facilitate individual adaptations and survive in changing environments (Mollá-Albaladejo and Sánchez-Alcañiz, 2021). However, explaining the proximate origins of changes in behavioral variability as a response to environmental challenges is not easy. Behavioral phenotypes emerge from many different levels of biological organization, including sensing of predators in the environment, adaptive gene expression, and even stochasticity in gene expression (Raj et al., 2010; Li et al., 2017; Honegger and de Bivort, 2018) to develop biases in idiosyncratic behavioral responses (Werkhoven et al., 2021) without changes in average left-right turning preferences.

This study found that flies reared with spiders were less mobile than control flies. Our recent study shows that predator stress during larval development of Drosophila impairs carbohydrate metabolism by systemic inhibition of Akt protein kinase, which is a central regulator of glucose uptake (Krama et al., revision 2, personal observation). This metabolic disorder is a likely cause of developing a diabetes-like biochemical and behavioral phenotype. An inability to metabolize glucose shifts the metabolism of fruit flies to triglyceride consumption, which decreases walking activity and might be a direct reason for the enhanced survival of fruit flies grown with spiders. Consistent with this idea, carbohydrate metabolism was found as one of the molecular functions most enriched in genes whose expression variation predicts variation in locomotor activity among individual isogenic flies (Werkhoven et al., 2021). However, the mechanism causing the higher variability of the turning behavior in flies with a diabetes-like phenotype remains unknown.

Antipredator behavior consists of a complex set of behavioral and physiological reactions and therefore likely involves neural pathways other than 5-HT. Honegger et al. (2020) found that both 5-HT and dopamine affect olfactory preference variability in fruit flies, and it is known that fruit flies can detect predators by their odors (Krams R. et al., 2021). Omura et al. (2012) and Stern et al. (2017) showed that the roaming speed of animals might depend on such neurotransmitters as tyramine, octopamine, npr-1, and daf-7, in addition to 5-HT. This suggests that future research on the neural regulation of antipredator responses in fruit flies should examine the effects of several neurotransmitters and their possible interactions. Experimental manipulations targeting more than one neuromodulator may be

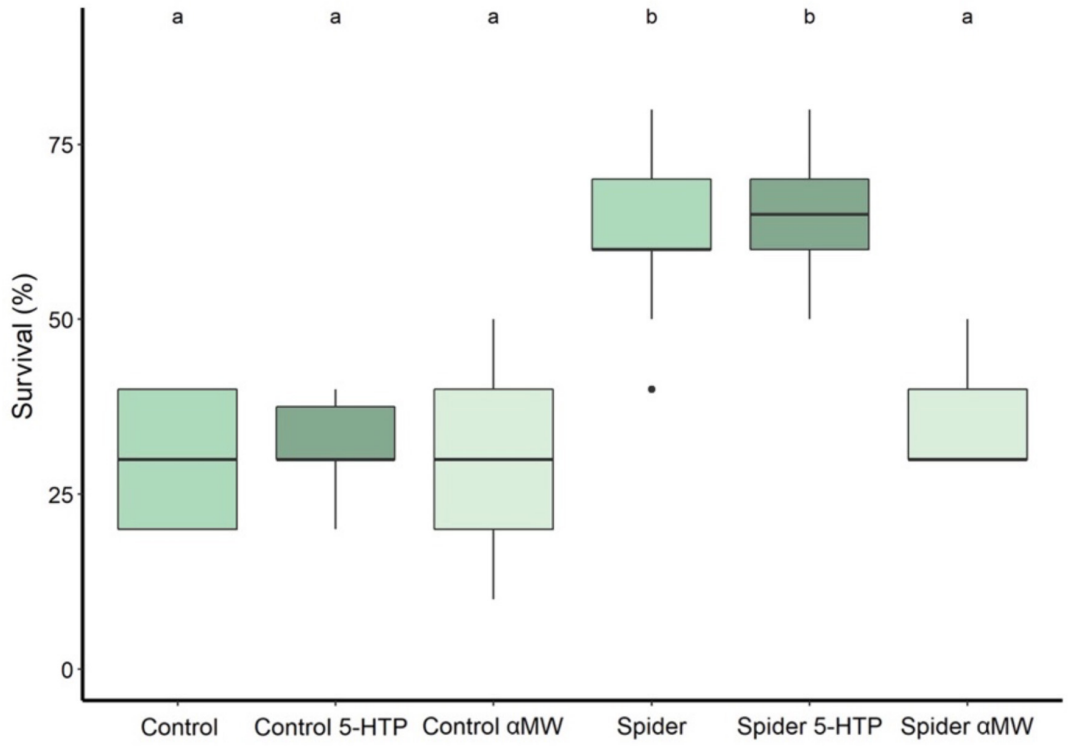

FIGURE 5
Survival percentage of adult fruit flies during a 12-h exposure to predation by jumping spiders. The flies reared with predators were exposed to predation during the larval stage; flies in the control group were raised without jumping spiders. Thick lines indicate the median, boxes show the Q1 and Q3 quartiles, and whiskers represent the upper and lower quartile, excluding outliers. Black dots represent outliers: data points more than 1.5 times interquartile range away from Q1 and Q3. Experimental groups that are not statistically significantly different (Tukey HSD, $P > 0.05$) are indicated by the same letter at the top of the figure.

essential, as one neuromodulator can alter the efficacy of other neuromodulators (Niederkofler et al., 2015; Niens et al., 2017). Finally, animals may respond to neuromodulators differentially based on their personalities (Krams et al., 2018). The complex interactions of neuromodulators and their behavior-specific effects on predictability will make this a rich and challenging area of research.

Data availability statement

The data that supports the findings of this study are available from the following Zenodo repository: https://zenodo.org/search?page=1&size=20&q=7936563.

Author contributions

TK, RK, and BB conceived and designed the study. TK, MM, RK, GT, SP, BB, and IK performed the study and collected and extracted data. MM, DE, GT, SP, and IK analyzed the data. TK, RK, TG, PJ, and KZ maintained stocks of experimental flies and spiders. ES, BB, and IK built the equipment. TK, MM, BB, and IK wrote the manuscript. TK, MM, RK, TG, GT, PJ, SP, KZ, DE, MR, ES, JC-G, BB, and IK participated in data analyses, results interpretation, and drafting the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was provided by the Latvian Council of Science to IK and TK (grants lzp-2020/2-0271, lzp-2021/1-0277, and lzp-2022/1-0348), the Estonian Research Council (Eesti Teadusagentuur; grant PUT1223). This study was also supported by the Fulbright Program of the U.S. Department of State. GT was supported by the Vilnius University Science Promotion Fund grant (MSF-JM-1/2021). BB was supported by a Sloan Research Fellowship, a Klingenstein-Simons Fellowship Award, a Smith Family Odyssey Award, a Harvard/MIT Basic Neuroscience Grant, and a National Science Foundation grant no. IOS-1557913. SP was supported by a grant of the European Social Fund (8.2.2.0/20/I/003).

Acknowledgments

We thank Dr. Kevin Cook at the Bloomington Drosophila Stock Center for providing us with stock flies. We are thankful to Don Cadle from Phids.com for sending us the spiders. We thank

Professors Christine R. B. Boake, Todd M. Freeberg, and Gordon M. Burghardt for their support during initial phases of this study and Professors Jae H. Park, Mariano Labrador, Ranjan Ganguly, and Joshua N. Bembenek kindly provided access to their lab facilities at Knoxville. We also thank Sudershana Nair and Kristers-Raivo Krams for their support at various stages of the study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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III PUBLICATION
III PUBLIKĀCIJA

PROCEEDINGS B

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Research

Cite this article: Krama T *et al*. 2023 A diabetes-like biochemical and behavioural phenotype of *Drosophila* induced by predator stress. *Proc. R. Soc. B* **290**: 20230442. https://doi.org/10.1098/rspb.2023.0442

Received: 23 February 2023
Accepted: 13 June 2023

Subject Category:
Ecology

Subject Areas:
biochemistry, behaviour, ecology

Keywords:
glucose, predation, stress, diabetes, serotonin, *Drosophila melanogaster*

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.6707580.

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A diabetes-like biochemical and behavioural phenotype of *Drosophila* induced by predator stress

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Predation can have both lethal and non-lethal effects on prey. The non-lethal effects of predation can instil changes in prey life history, behaviour, morphology and physiology, causing adaptive evolution. The chronic stress caused by sustained predation on prey is comparable to chronic stress conditions in humans. Conditions like anxiety, depression, and post-traumatic stress syndrome have also been implicated in the development of metabolic disorders such as obesity and diabetes. In this study, we found that predator stress induced during larval development in fruit flies *Drosophila melanogaster* impairs carbohydrate metabolism by systemic inhibition of Akt protein kinase, which is a central regulator of glucose uptake. However, *Drosophila* grown with predators survived better under direct spider predation in the adult phase. Administration of metformin and 5-hydroxytryptophan (5-HTP), a precursor of the neurotransmitter serotonin, reversed these effects. Our results demonstrate a direct link between predator stress and metabolic impairment, suggesting that a diabetes-like biochemical phenotype may be adaptive in terms of survival and reproductive success. We provide a novel animal model to explore the mechanisms responsible for the onset of these metabolic disorders, which are highly prevalent in human populations.

1. Introduction

Physiological, social and ecological factors can act as stressors capable of affecting the development of behavioural or biochemical phenotypes of organisms. Although stress responses often trigger adaptive physiological or behavioural changes by improving the survival of organisms, prolonged stress may harm the body by initiating life-threatening effects, impairing survival, and causing disease and death. Stress can aggravate various pathophysiological complications and metabolic changes in organisms [1]. Stress can also affect the nervous system of organisms and cause structural changes in different parts of their brains [2] through such processes as atrophy and decreased brain mass [3]. These effects may significantly impair memory, learning and cognition [4].

Mental disorders such as depression, anxiety and post-traumatic stress disorders (PTSD) are prevalent in human populations, affecting approximately 10% of people globally, a number that is steadily increasing [5]. The primary

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effects of metabolic disorders on quality of life are usually considered to be caused by adverse behavioural changes, leading to impaired emotional responses and loss of autonomy [6].

While several brain-specific metabolic alterations caused by these stress-related pathologies have been extensively studied, less is known about their effects on systemic metabolism [7,8]. This hypothetical connection, however, is increasingly coming to focus as numerous epidemiological studies have underlined the association of psychological conditions with dysfunctional glucose catabolization and insulin resistance [7]. Moreover, psychological stress has been implicated as a cause for the development of diabetes in initially healthy individuals [9–11]. These stress conditions do not, therefore, only impair cognitive functions and aggravate the clinical outcome of comorbid metabolic pathologies. Still, they are also potentially one of the factors increasing the growing prevalence of metabolic disorders. This view is supported by experimental rodent models, where chronic unpredictable mild stress and social defeat initiate metabolic dysregulation, leading to both peripheral and brain hyperglycaemia [12,13].

While mental stress induces numerous changes in both human and animal neuronal biochemistry and physiology [14–16], our understanding of the signalling pathway(s) connecting these alterations with general metabolism is fragmentary at best [17,18]. Much of the focus has been on the regulation of glucocorticoid hormone secretion released by the hypothalamic–pituitary–adrenal (HPA) axis [19]. These hormones control the systemic regulation of glucose utilization by inhibiting glucose uptake in muscle tissue and inducing gluconeogenesis in the liver from other molecules (e.g. branched-chain amino acids) [20–22]. This is believed to be an adaptation to conserve glucose for tasks such as the flight-or-fight response, increasing the odds of surviving an imminent attack [23,24]. However, prolonged activation of the HPA axis might become maladaptive, and its chronic upregulation is a well-defined consequence of psychological stress [25]. However, not all cohort studies demonstrate a connection between stress, glucocorticoids and metabolic dysregulation, suggesting that there are additional metabolic regulation pathways [26–28].

Here we test whether spider predation induces metabolic dysregulation in *Drosophila melanogaster* during larval development and whether the observed biochemical changes affect overall energy levels, locomotor activity, and survival of adult flies. We report that inducing predator stress in *D. melanogaster* causes systemic inhibition of Akt kinase, a central regulatory protein controlling glucose uptake in cells. At the organismal level, this leads to an impaired ability to metabolize glucose, suppressing glycolysis and shifting catabolism towards the utilization of fatty acids. Suppressing the consumption of carbohydrates has negative effects on locomotor activity and resistance to acute and chronic starvation. However, the findings of this study showed that predator-induced stress also resulted in increased survival in a predator-rich environment. Restoring normal metabolism either with serotonin supplementation or with metformin feeding negated the survival advantage. This indicates that metabolic reprogramming with long-term negative health effects may be adaptive by nature, sacrificing metabolic balance to enable escaping immediate death via, e.g. increased memory creation and, consequently, improved behavioural responses in the presence of direct threats to survival. Our work provides a novel understanding of how conditions similar to psychological stress can alter systemic energy catabolism and introduces a new animal model with exceptionally powerful genetics for future research on the topic.

2. Methods

(a) *Drosophila* husbandry and food formulations

Drosophila flies were reared in incubators at 23 ± 1°C under a constant 12 : 12 h light–dark cycle. This study used the wild strains Oregon-R-modENCODE (no. 25211) and w^{1118} of *D. melanogaster* obtained from the Bloomington Drosophila Stock Centre (IN, U.S.A.). The flies were isolated and sexed under carbon dioxide anaesthesia. To obtain *Drosophila* for this study, we placed 10 female and 5 male flies together to copulate and oviposit for 24 h in 24.5 × 95 mm vials (Genesee Scientific, San Diego, CA, USA). Each vial contained 18 ml of food. The food was prepared as a mixture of 500 ml water, 20 g dextrose, 15 g sucrose, 10 g brewer's yeast, 35 g cornmeal, 4.5 g agar and 12.5 ml 10% Tegosept (methyl-*p*-hydroxybenzoate) stock solution [29]. When required, metformin (Acros Organics, AC429720050) or 5-hydroxytryptophan (5-HTP) was added to the medium after cooling below 65°C, at concentrations of 20 mM and 5%, respectively.

The vials with eggs were placed horizontally on the floor of plastic jars (10 cm height × 12 cm diameter). Each jar in the experimental groups contained one pirate otter-spider (*Pirata piraticus*) collected throughout the spring/summer seasons. Flies remained together with spiders for their egg and larval stages [30]. The spiders were free to walk into the vials, where they often attacked and consumed *D. melanogaster* larvae.

Adult flies were collected within 5–7 h after the imaginal eclosion for biochemical analyses or used for behavioural assays within 2–3 days after eclosion. The flies used for biochemical investigation were frozen at −80°C.

(b) Feeding experiments

To measure the rate of feeding, food supplemented with blue dye (Blue FCF dye, Acros Organics A0373695, ThermoFisher Scientific) was fed to flies. The amount was quantified spectrophotometrically from homogenate. For each experiment, 140 flies from the control condition and 140 from the predator-stress condition were placed in two separate standard food bottles, and allowed to recover overnight from CO_2 exposure. On the next day, the flies were transferred without gas either to a new standard food or to food supplemented with 1% Blue FCF dye. After 1.5 h, 20 flies were collected and homogenized on ice by grinding in a mortar and pestle in 800 µl phosphate-buffered saline (PBS). Debris was pelleted at 10 000g_{max} for 10 min at 4°C, and 400 µl of each supernatant was transferred to 2 wells (200 µl each) of 96-well plates. Absorbance was measured at 650 nm, and values from lysates of flies kept on food without Blue FCF were used for background subtraction.

(c) Starvation tolerance measurements

In the chronic starvation tolerance test, flies were kept on 1% agar in tubes containing 10 individuals. Survival was monitored every 3 h. Death was determined as the last activity time point from the final recorded activity for each fly. In the acute starvation tolerance test, flies were starved on deionized water-soaked filter paper in tubes containing 10 individuals. The moisture content of the paper was controlled by injecting water with a syringe once a day.

(d) Survival of *Drosophila* under spider predation

To assess whether a diabetes-like phenotype has any adaptive value, we tested the survival of *Drosophila* under conditions of

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direct predation by spiders. We used 10 experimental and 10 control groups, each consisting of 10 male flies. We placed each group in a plastic container (20 cm width, 10 cm depth, 10 cm height) for 12 h during daylight time. Each jar contained one pirate otter-spider and one vial with *Drosophila* food (cornmeal, dextrose, sucrose, agar and yeast medium). We placed a layer of filter paper on the bottom of each container, and the top was covered by mash. The spiders were left without food for 12 h before the trials, while water was provided before and during the tests. Surviving flies were counted at the end of the experiment. Each otter-spider was used only once.

(e) Behavioural assays

We used sterile Petri dishes moulded from clear polystyrene (60 × 15 mm; Flystuff, El Cajon, USA) as novel arenas to record individual flies' locomotor activity. Only one fly was aspirated into the arena for each test. The locomotor activity of six flies was recorded with the resolution of 1920 × 1080 pixels at 5 frames per second simultaneously by a video-tracking system using the Logitech HD Pro Webcam C920 (Logitech Inc., Newark, CA, USA), fixed at a height of 25 cm above the arenas, and the software Debut Video Capture (NCH Software, Greenwood Village, CA, USA). To shorten the experiment duration, two identical video-tracking systems were prepared, which allowed tracking of 12 flies simultaneously. The video-tracking course was 15 min. We calculated the flies' average speed for each minute. The arenas were illuminated by reflected, diffused light from above by four MR 16 LED lamps (12 V, 6 W, 400 lm, 3000 K) located 0.9 m above the arenas. Illumination at the level of the arenas (3000 lux) was measured by a TES-1335 Digital Light Meter (TES Electrical Electronic Corporation, Taipei, Taiwan). All video recordings were made in the laboratory at between 21 and 22°C, and 35–40% relative humidity. Distance moved (start speed >0.20 mm s^{-1}; stop speed <0.20 mm s^{-1}) with the temporal bin width of 1 min as the most important locomotor activity parameter was extracted off-line from the recorded video files using EthoVision XT Version 11 software (Noldus Information Technology, Wageningen, The Netherlands). The distances moved were used to calculate the speed, representing the integral values of distances and time.

(f) Western analyses

Batches of 30 flies were homogenized with a pestle on ice in 300 µl of western lysis buffer (PBS with 1.5% Triton X-100) supplemented with protease and phosphatase inhibitor cocktails (Roche Complete Mini no. 11836170001 and PhosSTOP no. 04906845001) following the manufacturer's protocols. Lysates were incubated on ice for 15 min and then centrifuged at 13 000g_{max} for 15 min at 4°C to pellet debris. Supernatant protein concentrations were measured using the Bradford assay (Thermo no. 1856209), and 70 µg aliquots were loaded onto precast Bio-Rad Criterion AnyKD gradient gels. Gels were run in ProSieve EX running buffer (Lonza). Proteins were transferred to Amersham Protran nitrocellulose membrane (no. 10600020) in ProSieve EX transfer buffer (Lonza) at 35 V for 50 min in a BioRad Criterion Transfer chamber. Membranes were incubated in 5% BSA in 1× TBS/0.05% Tween for 1 h for blocking, after which they were incubated overnight at 4°C in the same buffer with primary antibodies. Antibodies and dilutions used were: Akt 1 : 5000 (Cell Signaling no. 9272), phospho-Akt 1 : 5000 (Cell Signaling no. 4054), ACC 1 : 5000 (Cell Signaling no. 3676), HRP-conjugated anti-rabbit 1 : 10 000 (PI-1000-1).

After washing membranes three times for 15 min with 1× TBS/0.05% Tween, they were incubated with anti-rabbit secondary antibody conjugated with horseradish for 1 h at ambient room temperature. After an additional three rounds of washing as before, results were visualized with the BioRad ChemiDoc XR detection system. For quantitation purposes, samples from control and predator-reared flies were run on the same gel with four individual biological replicates per group. When protein amount per lane was used for normalization, membranes were stained with Ponceau S solution (0.1% Ponceau S in 5% acetic acid), rinsed briefly with water, and documented using the BioRad ChemiDoc XR system. The signal was quantified, and the data were analysed with ImageQuant software. Western blots and corresponding Ponceau S-stained membranes used for quantifications are presented in electronic supplementary material, figure S1.

(g) Metabolite analyses

For carbohydrate measurements, 10 flies were homogenized in 400 µl of PBS and incubated for 5 min at 70°C. A total of 40 µl of lysate was transferred to four separate Eppendorf tubes with additions of 1 U of amyloglucosidase from *Aspergillus niger* (Sigma, total glucose measurement), 2× PBS (free glucose and background measurement) and 5 mU of porcine kidney trehalase (Sigma T8778, trehalose measurement). All reactions were incubated for 2 h at 37°C, after which they were briefly centrifuged, and 30 µl of supernatant was transferred to 96-well microtitre plates. One hundred microlitres of Glucose Assay Reagent (Sigma G3293) was added to all reactions except for one PBS-treated lysate mixed with 100 µl of PBS to measure the background signal. Reactions were incubated at 37°C for 30 min, after which absorption was measured at 340 nm. Free glucose, glycogen and trehalose were calculated by subtracting relevant backgrounds from measured values. A glucose standard curve was generated using 1 to 20 µg of glucose (per well). The results were normalized against protein amount measured with Bradford assay (Thermo no. 1856209).

For triglyceride measurements, 10 flies were homogenized in 800 µl of PBS with 0.1% Tween 20 and incubated for 5 min at 70°C. Twenty microlitres of each lysate was transferred to three Eppendorf tubes with additions of 20 µl of Triglyceride Reagent (Sigma T2449, total glycerol measurement) and 2 × 20 µl of PBS (free glycerol and background measurement). All reactions were incubated for 30 min at 37°C, then briefly centrifuged, and 30 µl of supernatant was transferred to 96-well microtitre plates. One hundred microlitres of Free Glycerol Reagent (Sigma F6428) was added to all reactions except for one PBS-treated lysate mixed with 100 µl of PBS to measure the background. Reactions were incubated at 37°C for 5 min, after which absorption was measured at 540 nm. Triglycerides were calculated by subtracting free glycerol from total glycerol measurement. A glycerol standard curve was calculated using 0.5 to 3 µg of glycerol (per well). The results were normalized against protein amount measured with Bradford assay (Thermo no. 1856209).

ATP concentration was measured using the ATP Determination kit (ThermoFisher Scientific). Thirty flies were homogenized in ATP isolation buffer (6 M guanidine-HCl, 4 mM EDTA, 100 mM Tris/Cl pH 7.8) and snap-frozen in liquid nitrogen, followed by boiling for 5 min. Debris was pelleted by centrifugation at 10 000g_{max} for 10 min at 4°C. Five microlitres of a 12.5-fold diluted supernatant was added to 100 µl of ATP Reaction Mix (Thermo Fisher; formulated according to the manufacturer's recommendations), and values were recorded using a Tecan luminometer with Greiner polypropylene plates (no. 655207). The results were normalized against protein amount measured with Bradford assay (Thermo no. 1856209).

Pyruvate was measured using BioVision kit no. K709 according to the modified protocol provided by the manufacturer. For pyruvate measurements, 20 flies were homogenized in 200 µl Pyruvate Assay Buffer on ice and then centrifuged at 10 000g_{max} for 10 min at 4°C. Fifteen microlitres of supernatant was mixed with 35 µl of Pyruvate Assay Buffer in a well of the

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96-well microtitre plate. Fifty microlitres of reaction mix (formulated according to the manufacturer's guidelines) was added to each well containing supernatant and incubated for 30 min at room temperature, after which absorption was measured at 570 nm. Parallel background reactions were performed by mixing supernatant with background mix, formulated according to the manufacturer's guidelines. The results were normalized against protein amount measured with Bradford assay (Thermo no. 1856209).

(h) Respiration exchange ratio measurements

Respiration exchange ratio (RER) was calculated as the ratio of CO_2 produced and O_2 used by flies. O_2 consumption in individual flies was measured by coulometric respirometry in a continuous O_2-compensating system at constant temperature and humidity (23°C and 55% relative humidity). Flies were placed into measuring chambers, and measurements were begun when the flies stopped moving and the minimum value of gas exchange was reached. CO_2 levels were determined using a LI-700 differential CO_2/H_2O analyser (LiCor, Lincoln, Nebraska, USA).

(i) Statistics

All measures (except for locomotor activity) are averages of four to ten biological replicates, and individual values are marked with red dots on diagrams. Each individual biological replicate measurement of metabolites, feeding and quantification of proteins on western blots represents 10–30 flies per sample, depending on the assay (see above). For the locomotory speed measurements, 24 (control) or 16 (predator-reared) flies were used. For metabolite, protein, feeding, RER, locomotion, and survival measurements, *p*-values were calculated using two-tailed Student's *t*-tests. Error bars represent standard deviations. In the case of locomotor activity measurements, nonlinear regression of one phase decay model was used: $Y = (Y_0 - \text{baseline})e^{-KX} + \text{baseline}$, where X is time, Y is a movement that starts at Y_0 and decays down to the baseline, Y_0 and the baseline have the same units as Y, and K is the rate constant equal to the reciprocal of the X-axis units. This model was fitted to the dataset using the least-squares regression method, and *p*-values were calculated for comparison of control versus predator-reared populations with the test. Survival was analysed using Mantel–Cox tests for the pairwise comparisons of the survival functions. In all cases, GraphPad Prism software was used to build graphs and calculate *p*-values. To check for a false-discovery rate, we performed the Benjamini–Hochberg test (electronic supplementary material, table S1). Numerical values for all tests, as well as other statistical parameters (d.f., chisq, *t*-statistics) can be found in electronic supplementary material, data.

3. Results

(a) Predator stress induces a catabolic shift towards lipid oxidation

Both carbohydrates and lipids, as key biochemical energy storage molecules, were measured in *Drosophila* Oregon strain flies reared with and without predatory spiders. While free glucose, its disaccharide trehalose, and polymeric form glycogen ($n = 8$) remained stable regardless of predator stress, triglycerides decreased, and free glycerol increased compared with controls ($n = 10$, figure 1*a*,*b*). This indicates increased utilization of lipids since lipolysis of triglycerides would provide free fatty acids for catabolism and simultaneously increase free glycerol concentration. Such specific loss of fat stores without any change in carbohydrate concentrations strongly indicates a shift in catabolism rather than inducing an overall starvation phenotype. Indeed, the RER ($n = 20$) of 0.76 in spider-reared flies supported this interpretation (figure 1*c*), indicating a firm reliance on a fatty acid breakdown in fuelling systemic ATP production.

(b) Predator stress reduces overall energy levels

Even if catabolism is re-oriented towards fatty acid oxidation, carbohydrates can contribute to this through de novo lipid synthesis. However, the levels of the rate-limiting enzyme acetyl-CoA carboxylase (ACC) controlling this process were decreased in flies experiencing predator stress ($n = 8$, figure 1*d* and electronic supplementary material, figure S1). Increased feeding intensity ($n = 14$), a typical response to resource scarcity in *Drosophila*, was not found (figure 1*e*). Complete reliance on only one type of catabolic fuel source caused a 20% decrease in steady-state ATP levels ($n = 10$, figure 1*f*). Not compensating for diminished ATP production by increasing food uptake or lipid synthesis must come at the cost of lower metabolism. Spider-reared flies were indeed observed to have lower speed than controls in walking/climbing assays ($n = 24$ and 16, figure 1*g*). Similarly, these flies were less resistant to both acute ($n = 220$) and chronic ($n = 274$ and 275) starvation, exhibiting shorter survival in conditions of limited food resources (figure 1*e*).

(c) Glucose uptake is inhibited

The activity of Akt, a central regulator of the conserved glucose uptake mechanism, is dependent on the phosphorylation state of threonine at its kinase domain and serine residue in its hydrophobic motif (at position 505 in *Drosophila* Akt), which was found to be significantly decreased in spider-reared flies ($n = 8$, figure 2*a* and electronic supplementary material, figure S1). This indicates reduced glucose transport, depriving glycolysis of its substrate and decreasing its end-product pyruvate ($n = 4$, figure 2*b*). Administering metformin, an anti-diabetic drug that facilitates glucose uptake in both humans and flies [32,33], restored the normal balance in the flies' carbohydrate/lipid usage ($n = 10$) and increased their RER ($n = 20$) to normal value (figure 2*c*).

(d) Serotonin complements metabolic dysfunction

The responses to external stimuli leading to different stress conditions are often mediated by changes in neurotransmitter levels. Serotonin dysregulation has been specifically associated with neurological stress that can cause several types of disorders in humans. In fact, w^{1118} strain flies with a mutation in the *white* gene and severely reduced serotonin levels compared with red-eyed strains [34,35] displayed a much stronger metabolic shift ($n = 10$, electronic supplementary material, figure S2). This could mean that serotonin mediates the effects of predator stress downstream from other parts of fly metabolism. We therefore asked whether elevated serotonin can alleviate predator-induced metabolic impairment. We fed flies with elevated concentrations of the serotonin precursor 5-HTP and analysed its effects on Akt phosphorylation ($n = 8$), pyruvate ($n = 8$), triglycerides ($n = 10$), free glycerol ($n = 10$), ATP ($n = 10$) and RER ($n = 20$) (figure 2*d*). In all cases, external administration of serotonin precursor restored these parameters in spider-reared flies to control levels,

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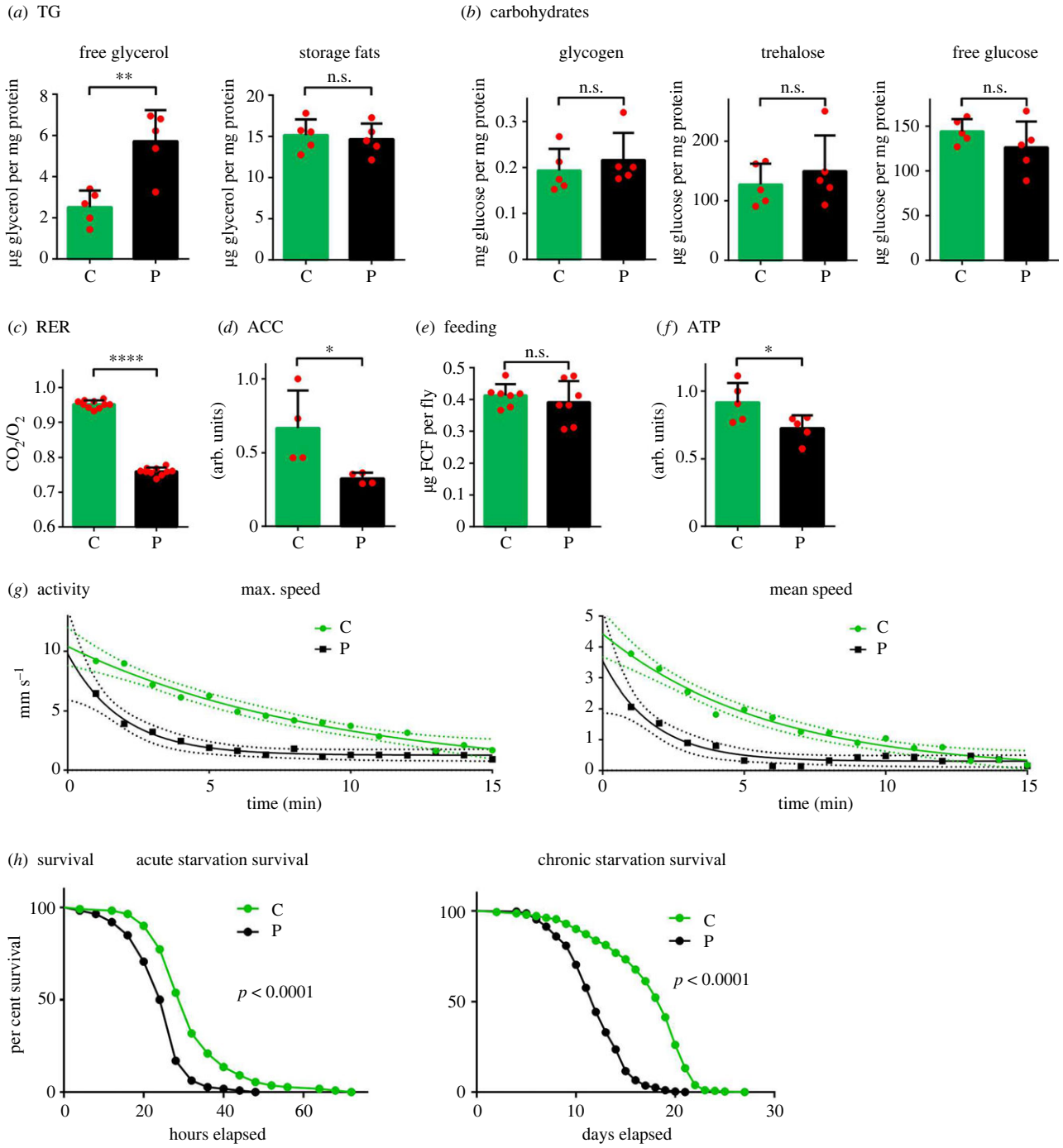

Figure 1. Effects of predator stress on the metabolism, locomotor activity, and survival in flies reared with spiders (predators) or without spiders (control). (*a*) Levels of free glycerol and storage fats. Relative but not absolute values of control flies have been published before [31]. TG, triglyceride. (*b*) Levels of carbohydrates: glycogen, trehalose, and free glucose. (*c*) Respiration exchange ratio (RER). (*d*) Amount of acetyl-CoA carboxylase (ACC) quantified against Ponceau S-stained total protein (for western blots, see electronic supplementary material figure S1). (*e*) Uptake of food containing 1% Blue FCF dye (FCF). (*f*) ATP concentration. (*g*) Nonlinear regression of maximum speed and mean speed measured across 15 min; test *p*-values for both cases are below 0.0001. Dots represent averages of 16 (control) and 23 (predator) experiments. The *p*-values of independent samples *t*-tests are 0.00017 for mean and 0.0047 for maximum speed. Dashed lines represent 95% confidence intervals. (*h*) Survival curves of flies kept on agar food (acute starvation, log-rank test $p < 0.0001$) and on 1% sucrose food (chronic starvation, log-rank test $p < 0.0001$). In all cases: *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, n.s.—not significant. C—control, P—predator-reared.

suggesting that supporting serotonin synthesis is sufficient for countering these metabolic alterations.

(e) Survival of flies under predation

The aforementioned changes in metabolism and decreased locomotor activity led us to ask whether predator presence affects the survival of flies. We housed flies together with predatory spiders (10 male flies and 1 spider per group; 10 experimental groups in total) in a closed space and observed the survivability of flies over 12 h. Surprisingly, there was an apparent increase in the survivability of predator-reared flies over control flies (figure 2*e*). Remarkably, feeding metformin and a precursor of serotonin that reversed metabolic defects also decreased the survival of predator-reared flies to levels observed in the control group ($n = 20$). This demonstrates that the increased survival of flies in response to predator presence comes at the cost of metabolic health.

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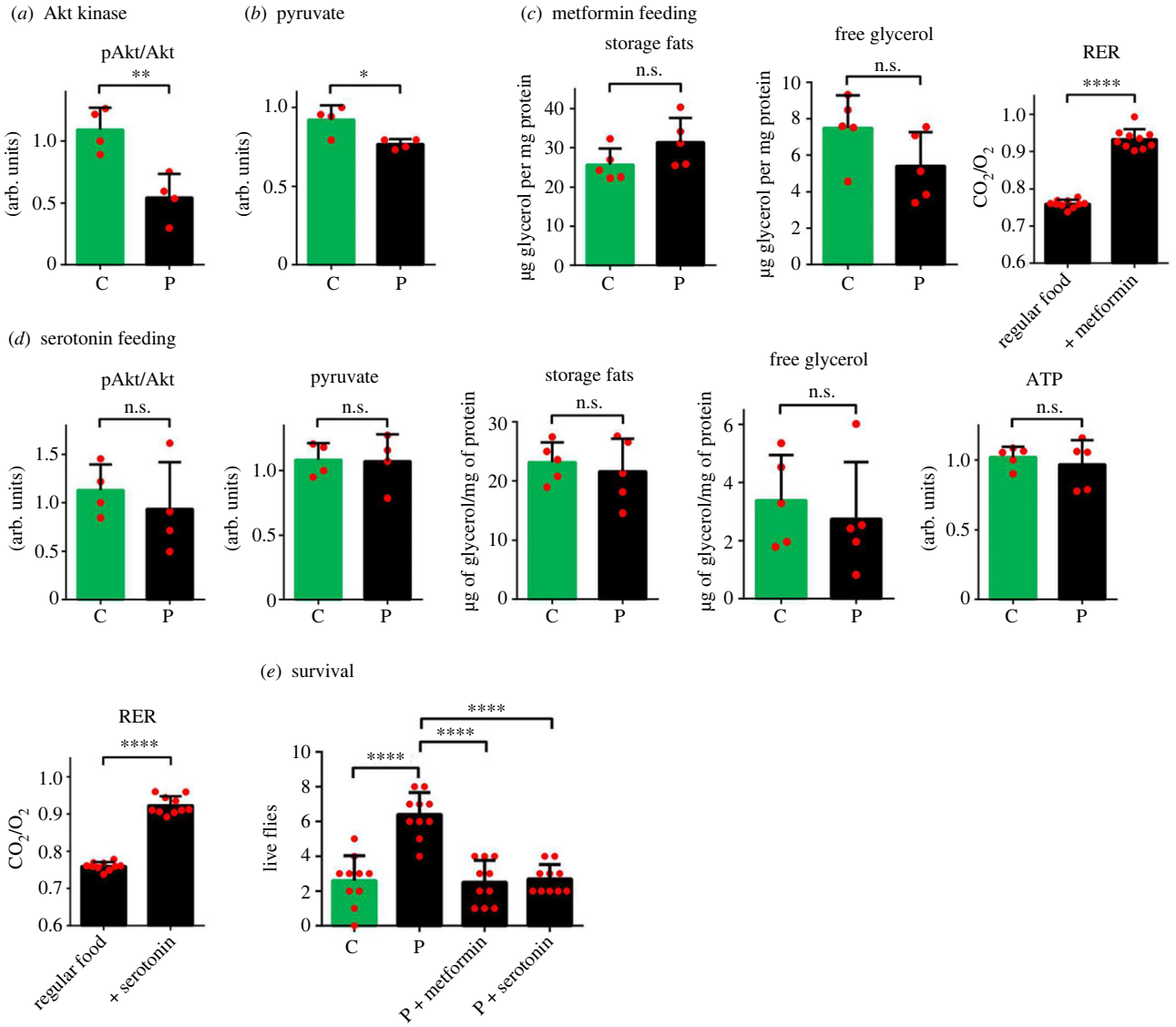

Figure 2. Effects of predator stress on metabolism/behaviour and pharmacological complementation in flies reared with spiders (predators) or without (control) spiders. (*a*) Phosphorylation of Akt kinase at Ser505. (*b*) Levels of pyruvate. (*c*) Effect of metformin feeding on storage fats, free glycerol, and predator-reared flies' respiration exchange ratio (RER). (*d*) Effects of serotonin feeding on Akt phosphorylation, pyruvate, storage fats, free glycerol, ATP and predator-reared flies' RER. (*e*) Survival of predator-reared and control flies with or without feeding 5-hydroxytryptophan (5-HTP) or metformin after 12 h of incubation with spiders. For better comparison, RER data of predator-reared flies from figure 1*c* are re-used in (*c*,*d*). In all cases: *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, n.s.—not significant. C—control, P—predator-reared.

4. Discussion

The effects predators have on prey are not limited to the death of prey individuals, but can induce a lasting condition of fear in the prey that survive in the presence of predators. As a result, prey often respond to predators in their environment by altering their morphological and physiological phenotypes during development [30,36–39]. Although these changes facilitate survival by improving escape abilities [40,41], predators may have enduring costly effects on prey individuals [42–44]. For example, predator-induced fear is one of the most common stressors employed in animal model studies of post-traumatic stress disorder [43]. This research has gained scientific interest because of the relevance of psychological stress in causing clinical depression and other metabolic disorders, such as type 2 diabetes, in humans. Although the underlying mechanism has remained unclear, increased serum glucocorticoid concentrations and catecholamine release are commonly associated with the development of insulin resistance [45]. Our results align with these findings by showing that *Drosophila* reared with predators develop a diabetes-like biochemical phenotype characterized by an inability to metabolize glucose, forcing a shift to triglyceride consumption. This is caused by a decreased activity of Akt kinase, a central regulatory kinase that has a major role in controlling glucose uptake. This protein facilitates a highly conserved glucose transport mechanism (e.g. via GLUT4-dependent pathway in muscles), and defects in this pathway are therefore closely associated with the development of diabetes [46]. Improving glucose transport using metformin, which has similar effects in flies to those in humans [33], restored the original metabolic balance in flies grown with predators.

Predator presence eventually changes the quality of the environment and affects the survival strategies of prey. While *Drosophila* flies rely on visual and olfactory cues for detecting predators such as spiders and mantises, it is currently unclear to what extent flies use separate sensory systems in different environmental conditions [30,47]. However, they do have a highly developed olfactory system that allows them to live for generations in complete darkness [48]. This sensory system is sufficient by itself to detect

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the presence of spiders, and even exposure to spider odours can elicit metabolic and developmental changes [30,49]. Regarding w^{1118} flies, it is also unclear whether they can use vision to detect predators, as they may have poor visual acuity [50]. However, w^{1118} flies easily chose walking corridors when tested in the Y-maze experiments [51], suggesting w^{1118} flies actively rely on vision while exploring their environment.

Although a number of studies have described how chronic stress can have enduring effects on metabolism and behaviour [43,52,53], the connection between neural chemistry and metabolism has remained unclear. Our finding that supporting serotonin synthesis antagonizes the described metabolic effects suggests a central role for serotonin in such biochemical communication. Serotonin has multiple biological functions: regulating courtship behaviour [54], affecting spatial memory [55] and olfactory learning [56], influencing phototactic behaviour [49,57], and affecting turning behaviour [58]. Furthermore, it participates in several pathways that overlap with the roles of other neurotransmitters, such as dopamine and octopamine (norepinephrine homologue in *Drosophila*). Owing to the variety of serotonin's roles in neural circuits, which are at least partially redundant, its effect on metabolism can be caused by a number of different mechanisms. One plausible explanation could be related to the observed interconnection between serotonergic and insulin-producing nervous systems. In *Drosophila*, serotonergic neurons are closely apposed with insulin-producing neurons, and these two neuronal systems communicate [59]. They control insulin signalling and, if defective, serotonin and insulin accumulate together, with suppressed peripheral insulin sensitivity. In humans, elevating serotonin has beneficial effects on metabolic balance, improving insulin sensitivity and glucose homeostasis [60]. This effect is relayed through serotonylation of the small GTPase Rab4, which elicits beneficial effects on glucose uptake, thus representing a convergence point between serotonin and insulin signalling. Since serotonin is decreased in human psychological disorders resembling the effects of predator stress, it is tempting to speculate a linear relationship between the metabolic reprogramming described here and serotonin levels. Tentative support for this hypothesis comes from a quantitatively stronger metabolic shift in the serotonin-depleted w^{1118} strain. However, serotonergic upregulation caused by the exogenous administration of serotonin might also elicit the observed reversion of metabolic changes.

Systemic effects on catabolism in predator-stressed flies resembled the effect of glucocorticoids in humans, a group of hormones released in response to stress conditions through activation of the HPA axis. These hormones antagonize the function of insulin by inhibiting the uptake of glucose in muscles and adipose tissue. They also downregulate glycolysis, inducing lipolysis and hepatic gluconeogenesis [20]. This mobilizes and reroutes energy reserves for specific tasks, e.g. increasing blood glucose levels to prepare the organism for a 'flight-or-fight' response.

Drosophila has no apparent neuroanatomical homologue of the mammalian HPA axis nor the same glucocorticoid hormones as humans. However, it has a central steroid hormone ecdysone, converted into 20-hydroxyecdysone (20HE) in haemolymph after its release. Best known for its role in inducing larval moults and metamorphosis [61], it also regulates metabolism by suppressing glucose use. Binding with its receptor (EcR) induces this protein's translocation to the nucleus, where it represses the transcription of genes central to glucose utilization [62]. This is antagonistic to the function of an oestrogen-like receptor (ERR) recently described as a receptor for glucocorticoids in *Drosophila*, suggesting an interplay with other steroid hormones in this organism [63,64]. The effects of 20HE are very similar to the deletion of ERR, which blocks the use of carbohydrates as a fuel source, leading to a shift towards lipid oxidation and depleting triglyceride reserves [64]. Furthermore, 20HE acts as a stress hormone in flies, upregulated in response to adverse environmental conditions and stressful social interactions [65].

Intriguingly, we found that predator stress enhances the survival of spider-reared flies in the adult stage when kept together with the spiders. This effect correlated precisely with metabolic reprogramming since the administration of metformin and serotonin precursor reverted the survival advantage to control levels. This indicates that this metabolic reprogramming is adaptive and provides a clear survival benefit at the expense of reduced metabolic fitness. One explanation for this finding is associated with the speed of movement and overall locomotor activity of the flies. Another possibility is linked to the glucocorticoid stress effect on memory. Stress-induced glucocorticoid release enhances memory consolidation and long-term memory in humans [66]. The effect is the same in flies, with ecdysone having a clear beneficial impact on long-term memory formation [65,67]. It is believed that these effects of glucocorticoids are linked to the conservation of glucose for neural tissue function, which is a primary carbon source. This adaptation fuels increased neural activity, especially learning and memory [68]. Brains are metabolically costly organs, as is the process of creating new memories (e.g. via increased synaptic connections) [69]. In fact, elevated carbohydrate uptake in humans and animals, including *Drosophila*, has an apparent memory-enhancing effect, especially for long-term memory [70–74].

In the aggregate, the results of this study allow us to propose a model explaining how chronic psychological stress, such as predator stress, induces metabolic disorders. Shunting glucose away from catabolically active tissues like muscle to be consumed by neurons is likely an adaptation to create memories and prepare for similar stressful conditions in the future. However, when stress persists and leads to chronic activation of the HPA axis and sustained glucocorticoid release, it will impair normal glucose metabolism and permanently shift systemic catabolism towards lipid oxidation, preventing the use of carbohydrates. Such loss of metabolic flexibility, especially in animals that use carbohydrates as the main form of energy source, will inflict fitness costs, leading to decreased ATP production and downstream effects on resistance to nutritional scarcity and locomotor activity. Therefore, this chronic activation of a mechanism that provides short-term benefits will cause decreased fitness if stress persists. This is supported by the observation that chronic activation of ecdysone signalling, although beneficial for an immediate response, can cause negative long-term effects [75].

The results of this study suggest that although the diabetes-like phenotype induced by predator presence reduces general health, it might be beneficial for survival. The insulin-producing system in *Drosophila* and other invertebrates

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differs to some extent from that of vertebrates, including humans. *Drosophila* flies have eight insulin-like peptides [76], which likely have different and partially overlapping roles in metabolism regulation [77]. This shows that insects may have numerous ligands for one receptor, while mammals have receptors with somewhat redundant functions but a restricted number of ligands. Also, while the effect of extra 5-HTP in increasing serotonin is straightforward, it might affect concentrations of another neurotransmitter. Tryptophan is a precursor of biopterin [78], a cofactor associated with serotonin and dopamine synthesis. While the metabolic shift in serotonin-depleted w^{1118} flies compared with the Oregon strain provides tentative support for decreased serotonin concentration in response to predator stress, neuron-specific measurements are required to fully understand the mechanism underlying this hypothetically adaptive metabolic shift.

Finally, metabolic disorders are often associated with the impairment and loss of dopaminergic function [79]. Predator-induced stress affects the levels of brain dopamine, which are decreased in rats exposed to predator stress [80,81]. Since the w^{1118} strain has reduced dopaminergic activity, the interconnected serotonin and dopamine pathways should be studied simultaneously in predator-induced stress. These numerous aspects must be considered to fully understand the role of stress in the development of metabolic phenotypes and similarities/dissimilarities of stress perception in humans and *Drosophila* [82].

Data accessibility. All data with individual numerical data points are available in the figures and in electronic supplementary material, data file [83]. The 'Material and methods' section contains all information necessary for replication of experiments.

Authors' contributions. T.K.: formal analysis, funding acquisition, investigation, methodology; D.B.: data curation, formal analysis, investigation; L.O.: formal analysis, investigation; S.P.: formal analysis, investigation; V.B.: formal analysis, investigation, methodology; I.P.: formal analysis, investigation, methodology; R.K.: formal analysis, investigation; E.M.: formal analysis, investigation; A.M.: formal analysis, investigation; M.J.R.: formal analysis, investigation; I.K.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, writing—original draft; P.J.: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, writing—original draft.

All authors gave final approval for publication and agreed to be held accountable for the work performed herein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This work was funded by the Estonian Research Council (Eesti Teadusagentuur) grant PUT573 to P.J. as well as Estonian Research Council grant PUT1223 and Latvian Council of Science (Latvijas Zinātnes Padome) grants lzp-2018/1-0393, lzp-2021/1-0277 and lzp-2022/1-348 to I.K. and T.K. S.P. was supported by the European Social Fund grant (Nr.8.2.2.0/20/I/003). I.K. was supported by Daugavpils University research grant 14-95/2023/20.

Acknowledgements. We are grateful to Eric Dufour, Lauri Saks, Maido Remm, Ülo Maiväli, and Kaspar Reier for help with statistical analysis. We thank Dr Kevin Cook for providing us with stock flies. Professor Benjamin de Bivort kindly commented on earlier versions of the manuscript. Dr Severi Luoto kindly improved the style and language of this article.

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Behaviour 161 (2024) 133–148

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Explaining the survival of the sickest: altered walking patterns are linked with improved adult survival in *Drosophila melanogaster* grown with predators during larval development

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Received 10 July 2023; initial decision 7 August 2023; revised 7 November 2023; accepted 8 November 2023; published online 2 January 2024

Abstract

Stress caused by predator exposure can lead to various behavioural, physiological, stoichiometric, and biochemical changes in prey. Prior research has shown that growth under predation stress can

Published with license by Koninklijke Brill NV | DOI: 10.1163/1568539X-bja10254
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cause the development of a diabetes-like biochemical phenotype in fruit flies. Exposure to predator risk during larval development decreases flies' walking activity, improving their antipredator strategies. However, it is unclear which elements of walking behaviour make flies less conspicuous to predators. This study shows that fruit flies ($N = 729$) grown with spiders walk shorter distances, accelerate faster and spend more time in a state of motion without movement (i.e., stomping in place) than control flies ($N = 839$). Under predation risk, adult flies grown with spiders survived better than control flies. We suggest that motions without movement may resemble sickness behaviour for predators, which we propose as the main reason for their better survival under direct exposure to predator attacks.

Keywords
acceleration, diabetes, *Drosophila melanogaster*, predators, stress, survival, walking behaviour.

1. Introduction

Multiple stressors, such as diseases, resource limitations, climate change, and predation, determine life histories, personality types, habitat use, and activity patterns of living organisms (Fardell et al., 2020; Daversa et al., 2021). Predators are a ubiquitous part of ecological communities, shaping their prey populations. Predators can, directly and indirectly, impact the dynamics of prey populations and the survival strategies of individual prey (Bijleveld et al., 2015; Rinehart & Hawlena, 2020). Predators can induce physiological states of fear in prey (Lima, 1998), which cause long-lasting stress conditions affecting developmental strategies, reproduction, and survival (Indrikis Krams, 2000; Brown & Kotler, 2004; Clinchy et al., 2004).

Stress has been considered a major causal factor in the pathogenesis of human metabolic disorders, including obesity (Scherrer et al., 2018), type 2 diabetes (T2D) (Engum, 2007; Mommersteeg et al., 2012; Rotella & Mannucci, 2013), and other metabolic diseases (Kivimäki et al., 2023). Long-lasting predator-induced stress is one of the stressors used in animal model studies of human stress conditions, such as post-traumatic stress disorder (Zanette et al., 2019), and it can be potentially applied to other metabolic diseases of animals. Indeed, a recent study found that fruit flies (*Drosophila melanogaster*) grown with predators develop a diabetes-like metabolic alterations (Krama et al., 2023a). This suggests that the chronic stress caused by sustained predation may serve as a good model to study systemic metabolic reprogramming by human chronic stress conditions. The mechanism of developing a diabetes-like biochemical signature in fruit flies involves serotonin-mediated inhibition of central metabolic regulator Akt

kinase with associated effect of limiting carbohydrate use (Krama et al., 2023a). Previous work has indeed demonstrated increased glucocorticoid and catecholamine concentrations to be associated with insulin resistance (Beaupere et al., 2021). While stress requires high carbohydrate intake to fuel behavioural reactions (Trakimas et al., 2019), in fruit flies, development under predation risk caused almost complete shift towards the use of lipids as a fuel source with reduced ATP synthesis and decreased locomotor activity (Krama et al., 2023a). Although the observed diabetes-like biochemical phenotype made flies sick, this surprisingly improved fruit fly survival under direct predation by spiders (Krama et al., 2023a).

Movement is a defining characteristic of life and critical for the survival and fitness of living organisms (Liedvogel et al., 2013; Honegger & de Bivort, 2018). Krama et al., 2023b suggested that lower locomotor activity makes flies reared with predators less conspicuous to spiders than control flies, which have higher locomotor activity. This might be one possible reason for beneficial effect on survival, however, it is unclear what causes the anti-predator benefits of reduced locomotor activity in predator-affected flies. Improved survival can be potentially achieved by shorter walking distances, slower walking speed, having more numerous and longer stops, and/or by the differences in the acceleration of movements. For example, a quick and more accelerated walk may help prey avoid a risky spot sufficiently faster than a long walk at a constant speed.

In this study, we investigated differences in walking behaviour and survival between fruit flies grown with spiders and flies grown without predators. We hypothesized that exposure to predator risk during larval development decreases flies' walking activity, promoting antipredator strategies. Accordingly, we first predicted that flies grown with spiders might interrupt their walks more often because their energy reserves need to be replenished as these flies rely on fewer fat reserves (Krams et al., 2016). We also predicted that fruit flies reared with spiders would accelerate faster at the beginning of each movement since their bodies contain more nitrogen (N), suggesting larger muscle mass (Krams et al., 2016). Greater predation risk is often negatively associated with the amount of fat reserves (Krams, 2002; Almbro & Kullberg, 2012). Thus, the survival of fruit flies grown with spiders (low fat reserves) during the larval stage was expected to be higher than in control flies grown without predators.

2. Materials and methods

2.1. Fruit flies

In this study, we used Oregon-R-modENCODE(#25211) wild strains obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN, USA). Fruit flies were kept in humidity-controlled climate chambers Panasonic MLR-352H (Panasonic Healthcare Holdings, Tokyo, Japan) at Daugavpils University at 25 ± 1°C, approx. 40% humidity, and a constant 12:12 light-dark cycle using white ambient LED illumination. To obtain populations of fruit flies, ten F0 males and ten females were placed in one polystyrene vial (Genesee Scientific, El Cajon, CA, USA) with fresh food for 24 h for oviposition. Test tubes with eggs were then placed in a free, ventilated container.

After the flies eclosed, to ensure virginity, they were extracted every 5–7 h from the containers using a weak piston pump LLG-uniVACUUPUMP 1 (Lab Logistics Group, Meckenheim, Germany) and carbon dioxide anaesthesia. Flies were separated by sex, and only males were used for this research since a large portion of female bodies is composed of eggs and reproductive tissues. This may affect body mass, metabolic processes, and possibly predator preferences (Burggren, 2017). Flies were also selected according to the time of eclosion: only individuals with a "normal" developmental speed, i.e., those eclosed 10–12 days after oviposition, were used.

All flies removed from the containers were transferred to 24 × 95 mm tubes with fresh food. The diet was prepared according to a recipe adapted from the Cold Spring Harbor Protocols (Lewis, 1960): 100 ml water was mixed with 4 g dextrose, 7 g cornmeal, 0.9 g agar, and 2 g of deactivated yeast. Tegosept (methyl-p-hydroxybenzoate, 10%; Genesee Scientific) stock solution was added to the food to inhibit mould growth. The finished food tubes contained approx. 9 g of cooked food, abundant enough to feed the larvae.

The density of F1 first-instar larvae across the vials was similar, and we averaged the density to 100 larvae/vial by removing extra individuals with a brush (Krama et al., 2023a). One test tube with laid eggs was horizontally placed in a plastic container (110 × 90 × 120 mm). In the experimental group, one common wolf spider (*Pardosa pullata*) was placed in each container. The spiders could freely enter the test tubes and attack the *Drosophila* larvae.

All fruit flies were subjected to experimental procedures within three days after eclosion. A total of 839 males were included in the control group and 729 males in the predator stress group.

2.2. Experimental design

A plate with Y-shaped mazes was made for this study (Buchanan et al., 2015; Krama et al., 2023b). Each plate consisted of two layers: the first layer was made of solid transparent plastic; the second layer, with 60 mazes carved into it, consisted of black matte plastic to reduce light reflections. Each maze consisted of three sleeves equally spaced 120 degrees apart, each 3 mm wide and 12 mm long. Each arm ended in a circular turn with a diameter of 5 mm. Each maze was individually closed by a triangle of thin glass projecting above the plate surface. The glass was coated with Sigmacote (Sigma-Aldrich, St. Louis, MO, USA) to make it slippery and prevent the flies from turning upside down and walking on the ceiling. The height of each maze was 2 mm. In this way, all the flies had enough space to move freely but could not flip over and reduce their speed because of insufficient adhesion to the surface of the glass. The plate with mazes was illuminated from below through a thick matte plastic to create a contrasting surface for further recording of the movements of each fly. The recording was done in darkness to avoid the light reflections on the glass, which would have obstructed an accurate analysis of the movements. A Basler Ace camera with a 1/1.8″ sensor (Basler, Ahrensburg, Germany) and Kowa F1.6/4.4-11 mm optics (Kowa Optimed Germany GmbH, Duesseldorf, Germany) was mounted above the plate. Custom settings were chosen to ensure the highest accuracy and lowest distortion.

The study was conducted at 22 ± 1°C and relative humidity of approx. 40%.

2.3. Mobility parameters of fruit fly walks

Each fly was gently placed in one maze, using a short carbon dioxide anaesthesia. All flies were given at least 25 min to adapt after awakening. This was followed by two hours of continuous recording of the walking behaviour of fruit flies. Each fly only participated in one trial. The video files were subsequently uploaded to Noldus EthoVision XT v.15.0 (Noldus Information Technology, Wageningen, The Netherlands) and analysed using the following parameters: Distance Moved (mm), High Acceleration State frequency

(see below), Maximum Acceleration (mm/s^{-2}) and Motion Without Movement frequency. These are the most important parameters by which insect movement patterns can be characterized (Winberg et al., 1993; Russig et al., 2003; Nilsson & Renshaw, 2004). For each metric, data was obtained as a mean value per individual fly.

The Acceleration metrics were used to mark bursts of rapid movement. The High Acceleration State was observed when the average acceleration of the object exceeded the 2.5 mm/s^2 threshold. The threshold value was adjusted by using the EthoVision XT Integrated Visualization tool. We used averaging interval of 2 to remove the effect of random changes in velocity between consecutive samples that would result in false transitions to High Acceleration State. The optimal state duration threshold was defined as 0.5 s and was found using the Integrated Visualization plot (i.e., we did not consider accelerations with a duration of less than half a second). It was used to filter out false readings from the body-point jitter that can be introduced by camera vibrations or minor body motions. The frequency of the High Acceleration state is presented as the median of all values for each group. Readings were recorded for the entire duration of the experiment.

Maximum Acceleration is presented as the median of all values for each group. Before calculating the acceleration, we ensured that the proportion of lost samples was less than 1%.

Distance Moved was determined within 2 h periods. We used a sample rate of 6 data points (according to Noldus). Higher values can lead to false readings and overestimation of the covered distance. On the other hand, small movements of the animal central point may be missed due to lower values (Pham et al., 2009).

2.4. Fruit fly motions without movements

Motion Without Movement ("Mobility" in the Noldus software) describes the degree to which an object's body moves without regard to the spatial displacement of the central point. This implies that measurements are taken only when there is no movement of the animal's central point in the horizontal plane. Drosophila flies often perform "stomping in place" type behaviours. To describe this motion, calculations do not require x and y coordinates but instead, use the change in the position of individual pixels. This is an important parameter to estimate the degree of an animal's motion regardless of its locomotion along the x and y axes. A classic example of this parameter is

animal grooming: although the animal's limbs and body are busy, the animal remains in one place.

We estimated the frequency of Motions Without Movements ("Highly Mobile" according to Noldus) using a threshold of 50% change in the pixel area of the detected subject. We used the default Averaging Interval set to 1 data point, which means that the measures are not smoothed before determining values.

One limitation of Motion Without Movement is that it directly depends on the number of pixels that compose the object under examination and, consequently, on the camera resolution. *Drosophila* is a small object consisting of approx. 100 ± 20 pixels, so we set an extremely high Immobility threshold of 50%. This means that the animal's motion was counted only if 50% of the pixels changed their position. In this way, we excluded the probability of recording false readings. To avoid false readings, we do not report the Immobility metric here.

2.5. Survival of fruit flies under predation risk

To assess whether growing up in the presence of spiders has any adaptive value for adult flies that survived the spider's presence, we tested the survival of *Drosophila* under conditions of direct predation by *P. pullata* as done previously (Krams et al., 2016; Krama et al., 2023b). In brief, we used ten experimental and ten control groups, each consisting of 10 male flies. We placed each group in a plastic jar ($20 \times 10 \times 10$ cm) for 12 h during daylight. Each jar contained one wolf spider and one vial with *Drosophila* food (cornmeal, dextrose, sucrose, agar, and yeast medium). We placed a layer of filter paper on the bottom of each jar, and the top was covered by mash. The spiders were left without food for 12 h before the trials, while water was provided before and during the tests. Surviving flies were counted at the end of the experiment.

2.6. Statistical analyses

The data were analysed using R (version 4.1.0). We used generalized linear models with gamma distribution to determine how the treatments during the larval stage (Spiders vs. Control) affect the Distance Moved by flies (mm), and Maximum Acceleration (mm/s^2). For High Acceleration State frequency and Motion Without Movement frequency we fitted generalized linear models with quasi-Poisson distribution and logit link function. Before

fitting the models, data of High Acceleration State frequency, Maximum Acceleration (mm/s^2), and Motion Without Movement frequency were natural log-transformed to reduce heteroskedasticity. To assess fly survival under predation between the treatments, we fitted a generalized linear model with binomial distribution and logit link function, setting proportion of survived flies as a response variable, and treatment during development as a fixed factor.

We considered the differences statistically significant at $p < 0.05$ in all tests. In addition, the Lowess track smoothing method (Hen et al., 2004) was applied when exporting data from EthoVision XT.

Statistics were visualized using GraphPad Prism (version 9.5).

3. Results

We found significant ($\chi^2_{1,1568} = 13.00$, $p = 0.003$) differences in the distance travelled: the flies of the control group covered longer distances (5039 $\pm$ 3517 mm; mean $\pm$ SD) within a 2-h period than the flies of the experimental group (4403 $\pm$ 3443 mm) (Figure 1A).

There were significant differences in frequency of entering the High Acceleration State ($\chi^2_{1,1568} = 53.376$, $p < 0.001$), and in Maximum Acceleration ($\chi^2_{1,1568} = 119.82$, $p < 0.001$) between the groups. Flies of the control group entered the High Acceleration State less often (4781 $\pm$ 1474 times; mean $\pm$ SD) than fruit flies raised with spiders (5746 $\pm$ 1823 times) (Figure 1B). The flies of the control group exhibited lower speed during accelerations (7.807 $\pm$ 5.665 mm/s^2; mean $\pm$ SD) than flies grown with spiders (9.829 $\pm$ 8.086 mm/s^2) (Figure 1C).

The control group had Motion Without Movement significantly less often ($\chi^2_{1,1568} = 19.183$, $p < 0.001$) (828 $\pm$ 476 times; mean $\pm$ SD) than the group raised with spiders (1005 $\pm$ 654 times) (Figure 1D). This shows that flies raised with spiders exhibited more "stomping in place" movements.

We found that flies grown with spiders survived the 12-h experiment significantly better ($\chi^2_{1,18} = 10.605$, $p = 0.0011$) than naïve individuals from the control group grown without spiders during their larval stage (Figure 2). On average, 1.6 $\pm$ 0.97 (mean $\pm$ SD) out of ten flies survived in the control group and 3.6 $\pm$ 0.97 (mean $\pm$ SD) survived in the group grown with spiders.

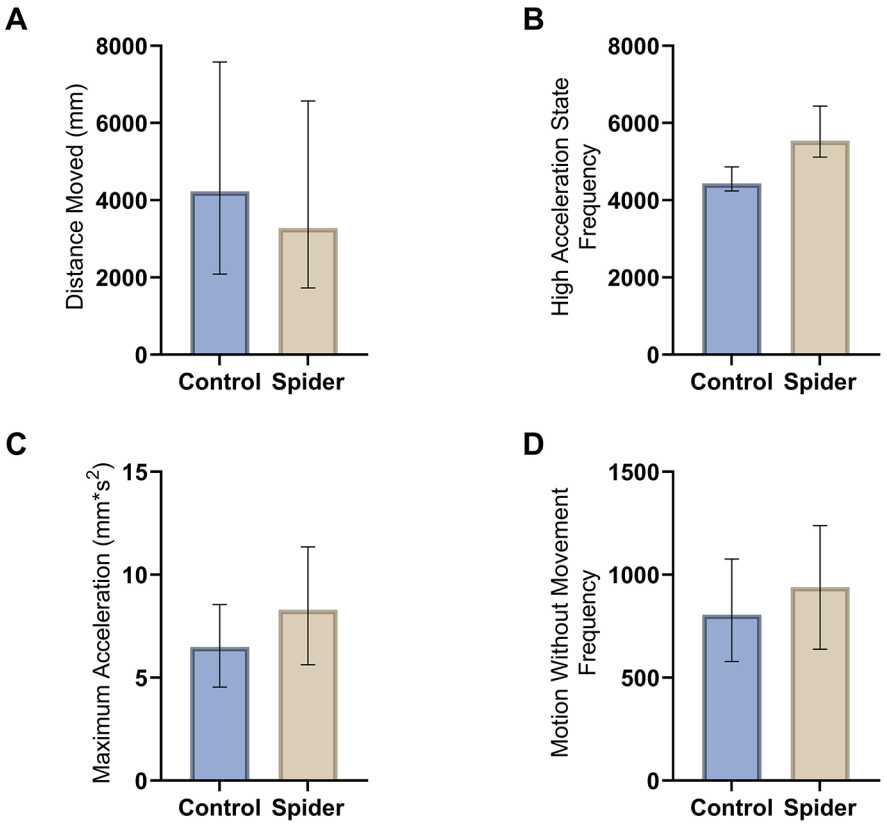

Figure 1. The median distance covered by the control group and flies grown with spiders within the 2-h period; the difference is significant at $p < 0.003$ (A). Difference between control flies and flies grown with spiders in the occurrence of Frequency of High Acceleration State; the difference is significant at $p < 0.001$ (B). Difference between control group and flies grown with spiders in the values of Maximum Acceleration; the difference is significant at $p < 0.001$ (C). Frequency of Motion Without Movement in the control group and in flies grown with spiders; the difference is significant at $p < 0.001$ (D). Error bars are ± SD.

4. Discussion

In this study, nearly 70 million data points were collected for *D. melanogaster* not exposed to spider presence during their larval development ($N = 839$) and those flies ($N = 729$) subjected to predation stress during their larval stage using a high-throughput data sampling method (Kain et al., 2012; Krama et al., 2023b). Short-term stress promotes oxidative stress and changes the metabolic balance away from anabolism and high-molecular-

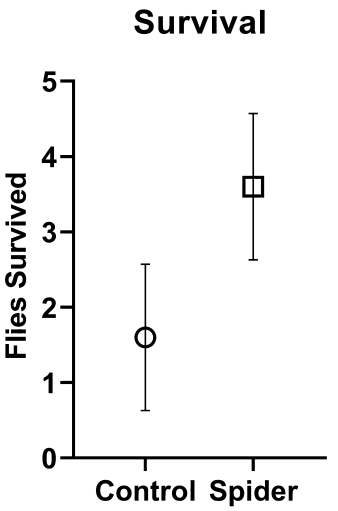

Figure 2. A mean number of ten control flies and ten flies grown with spiders surviving after a 12-h exposure to a spider. The difference is significant at $p = 0.0011$; Error bars are $\pm$ SD.

mass compound production, resulting in increased glycogen generation and hence a more significant requirement for carbohydrate intake (Trakimas et al., 2019). However, chronic psychological stress differs from short-term acute stress because prolonged stress, such as predator stress, may induce metabolic disorders (Krama et al., 2023a). As a result, the stress of encountering a predator early in life may alter an adult organism's phenotypic appearance, behaviour, and metabolism. Our results support earlier findings that walking activity is reduced in flies grown with spiders; a possible explanation for this is because diabetes-like metabolic disorder prevents fruit flies from using carbohydrates and shifts catabolism toward fat utilization (Krama et al., 2023a). Therefore, oxidation of lipids is expected to contribute proportionally more to major metabolic functions, including walking and flight movements in fruit flies grown with spiders than in control flies. Although fats are the most energy-rich macronutrient, fatty acids are a slower energy source than carbohydrates, requiring oxidative phopshorylation to generate ATP (Brosnan, 1999; Stryer, 1999). We show that fruit flies raised with spiders walk less while their initial movement acceleration is higher than in the control group, suggesting a more rapid exhaustion in flies grown with spiders.

In this study, we also confirmed that flies grown with spiders survived better in adulthood under direct exposure to predation risk than those from

the control group grown without any previous contact with predators. The flies affected by predation risk were observed to move in frequent and short dashes (Figure 1B). Importantly, their initial speed (acceleration) was substantially higher than that of flies of the control group (Figure 1C). We found that fruit flies from the control group moved at a more measured pace characterized by rare and low-intensity accelerations. Thus, the two groups of fruit flies radically differed in their movement pattern. Interestingly, during their rest stops, fruit flies reared with spiders moved their bodies (stomped in place) more often, which was found using the Motion Without Movement parameter (Figure 1D). Thus, fast accelerations, less distance walked, and distinctive "stomping in place during rest" behaviour may make fruit flies grown with spiders sooner to leave dangerous areas and become less attractive to spiders while resting between two subsequent walks.

The swift and sporadic stomping in place (Figure 1D) is a kind of unexpected behaviour of fruit flies grown with spiders. Instead of efficiently accumulating energy for the next series of walks, these flies spend their rest while quickly moving/shaking their bodies without spatial displacement. Despite being potentially more conspicuous to predators because of this activity, flies grown with spiders survived better than control flies when exposed to predators as adults. One explanation for this is that by turning in place and making small movements while staying in the same spot, these flies give predators false signals of their immediate future activities, such as flight initiation behaviour (Card & Dickinson, 2008).

Another explanation for the improved survival of flies reared with spiders is that the exposure of fruit flies to predators may cause metabolic disorders, and active motions without spatial displacement may reflect conditions of altered physiology, such as sickness behaviour characterized by a variety of coordinated symptoms such as anxiety, chaotic grooming behaviour, and failure to concentrate (Hart, 1988). It has been traditionally considered that predators are supposed to select substandard prey such as young, inexperienced, or sick individuals (Genovart et al., 2010). However, it has also been shown that some predators can non-randomly avoid infected prey (Hamilton & Zuk, 1982; Jones et al., 2005; Meyling & Pell, 2006). Although this strategy of predation has received much less attention in the literature (Gutierrez et al., 2022), our results show one more mechanism for the improved survival of sick animals expressing less predictable and more erratic walking responses than fruit flies without a diabetes-like biochemical

phenotype (Krama et al., 2023a). Previous research showed that fruit flies with a diabetes-like biochemical phenotype rely only on fat as a catabolic fuel source, causing lower body fat content (Krams et al., 2016) and a 20% decrease in ATP levels (Krama et al., 2023a). Also, fruit flies grown with spiders are known to have higher body nitrogen (N) content, suggesting increased muscle mass in these flies (Krams et al., 2016). Thus, higher body N and muscle mass, lower fat reserves, faster accelerations and faster exhaustion, more "stomping in place" behaviours, and lowered availability of ATP may explain more erratic and less predictable walking locomotion and better survival of fruit flies grown with spiders. Future research should test whether spiders actively avoid fruit flies with metabolic disorders and flies with infectious diseases and whether the behaviour of infected flies resembles that of fruit flies experiencing metabolic diseases.

This study shows that some conditions other than infectious diseases can make fruit flies unpreferred prey as individuals grown under sustained stress of predation survived better than control individuals when exposed to spider predation (Figure 2). Encountering stress during development and adulthood may lead to metabolic disorders, such as PTSD (Zanette et al., 2019) and diabetes-like phenotypes (Krama et al., 2023a), often affecting the nervous and endocrine systems. Although the link between psychological conditions and dysfunctional glucose catabolism has been established (Hackett & Steptoe, 2017), our understanding of the signalling pathways connecting environmental stress, behaviour, and biochemistry is rudimentary, and little is known about the impact of environmental stress on systemic metabolism. Based on the interconnections between physiology and behaviour, we would predict higher senescence rates of walking behaviour in flies grown with spiders. Eventually, even young fruit flies demonstrate a shift toward inefficient energy consumption at short sprints and an incapacity to cover long distances without accessible energy sources. Overall, a link between sickness behaviour and improved survival under predation risk looks tempting; however, future research on the sickness behaviour of fruit flies and other animals is needed because the underlying biochemical and behavioural mechanisms seem complex. Further studies on metabolism and movement of larvae, as well as the effects of senescence and their influence on behavior are also essential to develop a comprehensive interpretation of the observations.

4.1. Conclusion

In this study, we confirmed previous data indicating that larvae of *D. melanogaster* can detect danger in their environment, which changes the development of their adult behaviours to reduce predation risk. In the meantime, we supplemented the existing data with new and highly accurate observations of the movement of flies grown under spider predation risk. Although the movement patterns of fruit flies do not directly explain the enhanced survival of flies raised with spiders, they provide insight into the direction the behavioural changes occur. We suggest that there is a strong link between movement patterns, physiological stress, and systemic metabolism responsible for enhanced survival under predation risk.

Acknowledgements

We thank two anonymous reviewers for their valuable comments. The study was funded by the European Social Fund (grant 8.2.2.0/20/I/003) and Daugavpils University research grant Nr.14-89/11 to Sergejs Popovs. Tatjana Krama was supported by the Latvian Council of Science grants lzp-2020/2-0271 and lzp-2021/1-0277. Indrikis Krams was supported by the Latvian Council of Science grant lzp-2022/1-0348 and the Daugavpils University research grant 14-95/2023/20. Giedrius Trakimas was supported by the Vilnius University Science Promotion Fund (Grant No. MSF-JM-1/2021).

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