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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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

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.

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 $25 \pm 1°C$, 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 ($110 \times 90 \times 120$ 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 $22 \pm 1°C$ 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 $\pm$ 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-

Survival

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.

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