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Non-lethal effects of predation risk enhance long-term memory in *Drosophila melanogaster*

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Abstract

Predator fear can leave long-lasting impacts on the neural circuitry and behaviours of prey organisms, leading to enduring effects on memory characteristic of post-traumatic stress disorder. Previous research showed better survival in *Drosophila* grown with predators and, thus, stress. A better

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long-term memory can likely help *Drosophila* avoid visiting places where predators have been spotted before. We investigated the link between predator-induced stress exposure and memory retention in two groups of *Drosophila*. In this study, one group of *Drosophila* was exposed to visual and olfactory signals of spiders during the first five days of their adult stage, prior to memory testing. We found that 1 h short-term memory did not differ between experimental flies and flies in the control group, which were raised without spiders. In contrast, flies exposed to predator presence exhibited better long-term memory than control flies 24 h later. The strain of *Drosophila* used was found to possess a diabetes-like biochemical phenotype in a previous study, indicating metabolic shifts between glucose and lipids, which influences memory formation and retention. We show that linking long-term memory, body and brain metabolism, and predation risk-induced stress is needed to better understand the post-traumatic stress-associated biochemical and behavioural adaptations of *Drosophila*.

Keywords
Drosophila melanogaster, long-term memory, predation risk, stress.

1. Introduction

The traditional view in ecology is that predators impact prey organisms directly by killing them and reducing their numbers in the population (Lima, 1998; Lourenço et al., 2014). Yet, research has increasingly shown that prey species are also profoundly affected by simply perceiving the presence of predators within their environment (Lima, 1998; Suraci et al., 2016; Allen et al., 2022). Predators have been found to induce fear in their potential prey, affecting reproductive and survival strategies (Dudeck et al., 2017). This fear response can manifest in various physiological and behavioural changes in prey, such as alterations in social interactions, habitat choices, and general lifestyle to evade predators (Suraci et al., 2016; Potash et al., 2023). The lethal and non-lethal effects of predation are often costly to prey individuals (Sheriff et al., 2020). However, predator presence or cues of predators may have a positive impact on some prey individuals, such as priming prey reactions for subsequent encounters with similar threats (Krams et al., 2016; Krama et al., 2023a). This complex interplay underscores the intricate balance between fear and survival strategies in the face of predation.

Behavioural reactions like anti-predatory behavioural responses occur in response to external sensory stimuli, which are transduced along specific pathways of the central nervous system (CNS). Although stress can cause impairment to the development and connectivity of neural circuitry (Clinchy et al., 2011, 2013), subserving decision-making and behavioural expression of physiological and neurobiological states in the long term, in the

short term, these changes may be adaptive. For example, a recent study demonstrated that predators can induce a heightened sensitivity to predator danger, elevated neuronal activity in the amygdala and hippocampus, and improved long-term memory (LTM) in black-capped chickadees (*Poecile atricapillus*) that had been exposed to predator signals for only two days (Zanette et al., 2019). These neurobiological effects of predation risk-induced fear on black-capped chickadees resemble post-traumatic stress disorder (PTSD) in humans, which supplements the conclusions and assertions made by the growing body of literature researching the link between predation risk-induced stress and possible PTSD-like responses by non-human animals (Adamec et al., 2008; Cantor, 2009; Campos et al., 2013; Clinchy et al., 2013; Zoladz and Diamond, 2016; Zanette et al., 2019). These enduring memories suggest adaptive changes, as the respective CNS mechanisms allow prey to prioritize survival over other needs.

Like many prey species, the common fruit fly (*Drosophila melanogaster*) exhibits a strong response to the visual and chemical cues of predators (de la Flor et al., 2017; Krams et al., 2021a,b). Exposure to these predation cues during development leads to several adaptive changes: fruit flies are smaller at full maturation, they are faster in the negative geotaxis tests, their bodies contain higher concentrations of nitrogen (indicating more muscles and smaller amounts of lipid reserves), and their movements become more erratic and unpredictable (Krams et al., 2016; Krama et al., 2023a; Popovs et al., 2024). These morphological and behavioural changes might be caused by adaptive changes in fruit flies triggered by predator stress, leading to increased survival under direct predation by spiders (Krama et al., 2023b). The metabolic requirements of such changes entail a shift towards systemic use of lipids as fuel and a downregulation of glucose utilization (Krama et al., 2023b). This could be caused by a need to reserve glucose for neural tissue, where it is used for generation of LTM to better deal with these conditions in the future. In fact, enhanced LTM is induced in response to predator stress in other animals (Forest et al., 2016). However, despite these insights, the specific effects of predator-induced stress on enhancing LTM in fruit flies have not been directly explored.

In this study, we examined whether exposure to predators during enhances LTM in male fruit flies when they encounter spiders as adults. Stress is a significant factor in the development of human metabolic and mental health problems, such as type-2 diabetes, which is known to impair memory

(Arvanitakis et al., 2020). Severe stress from trauma like war, sexual assault, or aggression can generate PTSD in humans, and PTSD often has a cascade of affective, cognitive, and neural impacts on those suffering from it (Likitlersuang et al., 2023; Womersley et al., 2023). Recently there have been calls for researchers to make stronger experimental connections between such stressors and memory impairments (Dunsmoor et al., 2022). Since a recent study found that fruit flies grown with predators develop a diabetes-like metabolic phenotype (Krama et al., 2023a), we hypothesized that stress induced by predators might conversely supplement LTM in stressed fruit flies.

2. Materials and methods

2.1. Drosophila husbandry and predator exposure

The stock animals were maintained in a laboratory at the University of Tennessee, Knoxville, at 24 ± 1°C under a constant 12:12 hour (h) light–dark cycle. The wild strain Oregon-R-modENCODE (No. 25211) of *D. melanogaster* was used as the focal prey species, and wild adult (c. six months old) male jumping spiders (*Phidippus apacheanus*) were used as predators. This spider species is distributed across much of the US and depredates both larvae and adult flies (Edwards, 2004). The fruit flies were obtained from the Bloomington *Drosophila* Stock Center (Indiana, US). The spiders were collected in Florida, US, and they were received from the supplier phids.net. This spider is easy to house and breed in captivity (Krams et al., 2016).

In the predator exposure group, spiders were added to the growth chambers of flies ($N = 400$) (Plexiglas jars, 10 cm height × 12 cm diameter) on the day of their imaginal eclosion (Krams et al., 2016). The predators were separated from the flies with a mesh net in the middle of each fruit fly jar. We provided spiders with fresh water and 5–10 fruit flies every day. While flies could see and smell the predators, spiders could not kill the flies. Predators and prey were kept together until the fifth day of the fly adult stage, when we started memory conditioning of fruit flies. Control group flies experienced the same conditions and stimuli in their growth chambers as predator group flies, but with no spiders added. In this study, we used only males because a large portion of female bodies is composed of eggs and reproductive tissues. This can affect stress-related metabolic processes and possibly memory formation (Burggren, 2017).

2.2. Conditioning and memory tests

Conditioning (i.e., associative learning) and memory tests were performed following the methods of previous studies (Mery and Kawecki, 2005). We used samples of 10 adult flies, raised in standard conditions and aged 6–7 days. The conditioning procedure consisted of 5 training sessions separated by 20-min intervals (i.e., spaced protocol). During associative conditioning, flies were first exposed for 30 s to one odorant simultaneously with a mechanical shock of 2000 rpm vibration pulses of 1 s duration, delivered every 5 s by a test tube shaker (Heidolph Instruments, Schwabach, Germany). This period was followed by a 60 s rest period (no odour and no shock). Then, for 30 s, another odorant was delivered without shock. The training session ended with a second rest period of 60 s. 3-Octanol and 4-methylcyclohexanol (both 0.6 ml/l paraffin) were used as odorants (Mery and Kawecki, 2005). Each fly group was chosen to be conditioned randomly to either 3-octanol or 4-methylcyclohexanol. The results of the final trial (the fifth out of 5 trials, referred to as the "conditioning" group hereafter) of associative conditioning were used to characterize the learning of fruit flies.

We tested 1 h (short-term memory, STM) and 24 h memory (LTM) retention after associative conditioning (Tully et al., 1994; Margulies et al., 2005; Wang et al., 2023). During the memory retention assay, the flies walked to the choice point of a T-maze, in which they were exposed to two converging currents of air, one carrying 3-octanol and the other 4-methylcyclohexanol, and then allowed to choose between the two odours for 60 s. The memory score was calculated as the difference in the proportion of individuals choosing 3-octanol between flies conditioned to avoid 4-methylcyclohexanol and those conditioned to avoid 3-octanol. We conditioned 400 fruit flies in the predator exposure group ($N = 200$ STM flies, $N = 200$ LTM flies) and 400 fruit flies in the control group ($N = 200$ STM flies, $N = 200$ LTM flies); 800 flies in total. Each fly was used only for one memory test.

2.3. Statistics

We conducted the Shapiro–Wilk test to assess the normality of our data distribution. Since the p-value was <0.05 for all samples, we concluded that the data in all samples were not normally distributed, thereby justifying the use of a non-parametric approach. We ran Friedman's tests (corrected for ties) to assess the differences in memory retention percentage at three time

points (0 h 'conditioning'; 1 h 'STM'; and 24 h 'LTM') in predator exposure treatment and control groups of fruit flies. Wilcoxon pairwise tests with Bonferroni correction were used for post hoc analysis. We also compared the differences in memory retention percentage between predator treatment and control groups of fruit flies at three time points using Mann-Whitney tests with Bonferroni correction for multiple comparisons. Results are shown as median, min–max, and differences were considered significant if $p < 0.05$. The types of odours were excluded as potential factors in our models because the substances (3-octanol and 4-methylcyclohexanol) were randomly chosen during trials. Analyses were performed using Past 3.26 (Hammer et al., 2001).

3. Results

Mean memory retention (%) differed significantly between time points in control (Friedman test: χ^2 (tie corrected) $= 38.68$, df $= 2$, $p < 0.0001$) and treatment fruit flies (Friedman test: χ^2 (tie corrected) $= 38.68$, df $= 2$, $p < 0.0001$). Memory retention (%) diminished within both control (median, min–max; after the conditioning trial: 100%, 80–100%; STM: 80%, 80–90%; LTM: 20%, 10–30%; Wilcoxon tests: all $p < 0.001$) and predator treatment (after the conditioning trial: 100%, 80–100%; STM: 80% 80–90%; LTM: 35%, 30–40%; Wilcoxon tests: all $p < 0.001$) groups. It did not differ significantly between control and predator treatment groups after the conditioning trial and STM (Mann–Whitney tests: $p = 1.0$ and 0.535, respectively) (Figure 1). However, fruit flies from the predator treatment group had significantly higher LTM retention (%) than the flies from the control group (Mann–Whitney test: $p < 0.0001$), indicating lower memory decay in the predator treatment group (Figure 1).

4. Discussion

Our results show that the LTM retention of fruit flies in the predator treatment experimental group was superior to that of control flies, as the memory decay during 24 h was less in the flies exposed to spider presence. Enhanced LTM can offer a significant advantage in predator-rich environments, aiding in survival. An improved memory can help fruit flies better remember where predators are encountered most often to avoid visiting such places. A suggested biochemical mechanism is the release of glucocorticoids, or

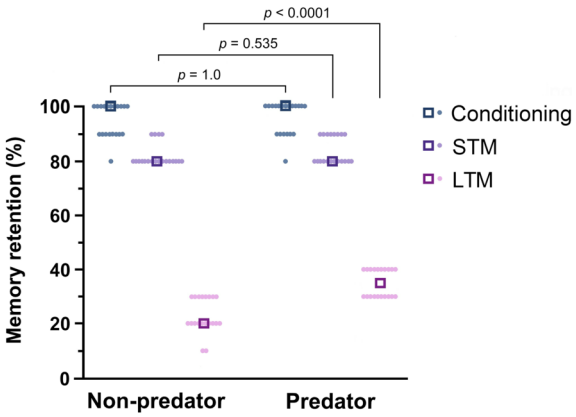

Figure 1. Differences between non-predator (control) and predator (spider) exposure treatment groups in their learning after the final conditioning trial (conditioning), short-term memory (STM), and long-term memory (LTM). Squares indicate medians, small circles individual data points; $N = 20$ for each treatment group. The p-values between respective treatment groups are presented. In both non-predator and predator groups, memory retention in the conditioning group was significantly greater than in STM, and memory retention in STM was significantly greater than in LTM.

their fruit fly analogue ecdysone, which can stimulate memory consolidation and LTM formation (Ishimoto et al., 2009, 2013; de Quervain et al., 2019). Another stress mechanism is octopamine release, as it is produced during stress (Adamo, 2022), and blocking octopamine receptors results in impaired learning (Segi et al., 2023).

To perform flight-or-fight responses under predation risk, body proteins are catabolized to produce glucose (Christianson and Creel, 2009; Hawlena and Schmitz, 2010a,b). However, predator presence is also known to induce a diabetes-like biochemical phenotype formation in fruit flies (Krama et al., 2023b). This is contradictory as it prevents glucose from being used as the main energy source to fuel stress responses. Importantly, while human diabetics cannot use glucose for their systemic metabolism, glucose remains the main fuel for the brain tissues (Li et al., 2023). Brain activity and especially memory formation are costly processes, as memory requires expression of specific genes (Hoedjes et al., 2015), extensive protein synthesis and expansion of synaptic connections (Burns et al., 2011; Wu et al., 2017). Thus, glucose might be conserved for neural tissue to increase its activity, including functions such as learning and memory (Wirth, 2015). It has been shown that elevated carbohydrate uptake in humans and in non-human animals,

including *Drosophila*, has an apparent memory-enhancing effect (Greenwood and Winocur, 2001; Lu et al., 2012; Plaçais et al., 2013; Totani et al, 2020; Chatterjee and Perrimon, 2021). Therefore, shunting glucose away from catabolically active tissues like muscle to be consumed by neurons could be an adaptation for facilitating memory creation and priming neurobiological responses for stressful conditions, such as predator presence. This would explain the clearly adaptive effect of flies with diabetes-like biochemical phenotypes having greater survival rates than flies that can use glucose for their systemic metabolism (Krama et al., 2023b; Popovs et al., 2024).

Long-lasting predator-induced stress is one of the stressors used in animal model studies of human PTSD (Adamec et al., 2008; Cantor, 2009; Campos et al., 2013; Clinchy et al., 2013; Zoladz and Diamond, 2016; Zanette et al., 2019). Our results demonstrate enhanced LTM in response to predator stress in a potentially disease-associated biochemical phenotype (Krama et al., 2023b), adding weight to the argument that the adaptive nature of this negative metabolic effect, stemming from shifts in energy source use and metabolism, is tied to memory formation. Our results also suggest that fruit flies are valuable for studying PTSD-like effects in animals due to the relative ease of studying the biological underpinnings of memory formation and subsequent behavioural consequences with them as model organisms.

Research indicates that long-term memory (LTM) formation is a complex process influenced by stress through the interaction of adrenergic agents and glucocorticoids released during stressful events. These hormones impact neural networks essential for memory generation (Roozendaal, 2002; Sandi and Pinelo-Nava, 2007; Goldfarb, 2019). Studies have confirmed that stress contributes to memory formation when DNA damage and inflammation are caused in hippocampal neurons, leading to long-lasting memories in mice (Jovasevic et al., 2024). Similarly, stress from predation risk induces comparable neural changes (Kotrschal et al., 2017; Mitra, 2019). Predator-induced stress offers a valuable model for studying LTM formation in mice and fruit flies, enhancing our understanding of the relationship between stress and memory at the neuroanatomical level and beyond.

It is known that *Drosophila* strains vary in regard to baseline neurotransmitter levels (Krams et al., 2021a,b) and, thus, baseline behavioural state (Maloney, 2021). For instance, white-eyed (w1118) mutants have greatly reduced serotonin levels (Borycz et al., 2008) and dopaminergic activity (Krama et al., 2023a) when compared to red-eyed strains. Stemming from

this discrepancy, white-eyed flies tend to have deficits in courtship behaviour (Lee et al., 2008), aggression (Hoyer et al., 2008), learning (Sitaraman et al., 2008), and locomotion (Xiao and Robertson, 2016). However, they are observed to display the diabetes-like phenotype as well (Krama et al., 2023b). Therefore, across fly strains, observing the roles of neurotransmitter level, baseline behaviour, propensity to display diabetes-like phenotypes, and predator species on stress-induced LTM retention is needed to support a robust link between neurophysiology and behaviour.

Drosophila go through four stages of development: embryonic, larval, pupal, and adult (Reaume and Sokolowski, 2006). Therefore, it is pertinent to examine fruit fly learning retention through the scope of developmental neuroscience. The pupal stage marks the beginning of metamorphosis in flies, involving a significant change in the neuronal structuring of the mushroom body (Truman et al., 2023). This may suggest that larval memories are not retained in adulthood. Though the mechanism of this developmental shift in neural circuitry is becoming understood, the resultant outcome of the shift on memory and behaviour needs to be studied. The associative learning and memories from olfactory cues that persist in *Drosophila* mushroom bodies (Hige, 2018) may still be retained through the metamorphosis reshuffling, as other insect species preferentially reproduce on the same species of plants where they initially matured (Blommers et al., 2004; Dion et al., 2019). We suggest observing whether fly larvae exposed to pheromones of spider species "X" during pre-pupal development are predisposed to naturally avoiding spider species "X" as mature flies when compared to flies not exposed to spider pheromones. Furthermore, it would be critical to assess whether fly larvae exposed to predator pheromones during pre-pupal development undergo a different mushroom body re-assemblage during metamorphosis due to stress, and to research what effect this might have on lifetime memory and associative learning outcomes, as well as overall fitness.

Interestingly, stress did not significantly affect conditioning and STM in this experiment. The nervous system in *Drosophila* utilizes acquisition, consolidation, forgetting, and retrieval in memory operations (Davis, 2023), with unconsolidated memories decaying within 6–8 h and consolidated memories lasting at least 24 h (Wang et al., 2023). Since LTM formation requires the synthesis of specific proteins and consolidation processes, and STM does not require consolidation (Roselli et al., 2021; Davis, 2023), our results might

suggest that predator-induced stress specifically affects memory-related protein synthesis and memory consolidation and does not affect STM because the latter does not rely on consolidation.

We recognize our findings are derived from the effects of one predator's presence on the behaviours of one *Drosophila* strain. Future studies should aim to evaluate the generalizability of our results to assess whether the behavioural changes we observed are predator-specific and/or *Drosophila* strain-specific. Fruit flies are observed to exhibit predator-specific antipredator behaviours in response to olfactory cues from multiple species of spiders which prey upon them (Kempraj et al., 2017). This study shows that foraging rates and escape behaviours varied depending on which spider species' olfactory cues flies were exposed to, supporting the idea that flies may not respond to all pressures of predation risk in a uniform behavioural pattern. Future studies are necessary to explore whether fruit flies can retain specific predator-related memories, the duration these memories last, and the underlying mechanisms of memory formation under predation risk.

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