How does learning about the spatial environment modulate the defensive responses of *Drosophila melanogaster*?

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Resumo

A capacidade de percepcionar e de responder a ameaças iminentes é crucial para assegurar a sobrivência dos animais e a prepetuação das espécies. Notavelmente, sabe-se muito pouco sobre como é que os animais selecionam comportamentos defensivos especificos (e.g. freeze vs fuga) e sobre como é que certas variáveis, tais como a familiaridade com o contexto espacial, modulam estas decisões.

Para estudar como é que a familiaridade com o contexto afecta a selecção dos comportamentos de defesa, apresentámos, a moscas da fruta, um estímulo designado de *looming* – uma sombra que se expande mimetizando um objecto de grandes dimensões em rota de colisão – num ambiente onde a fuga não era possível. Nestas condições, as moscas tipicamente correm or *freezam*. Deste modo, hipotetizámos que o nível de familiaridade com o contexto pode ter um impacto na seleção dos comportamentos de defesa. Para testar isto, analizámos os comportamentos defensivos da *D. melanogaster* exposta aos *loomings* num ambiente inescapável enquanto manipulávamos a expressão de genes relacionados com a memória e com a aprendizagem (*rutabaga*, *foraging* e *S6KII*).

Os nossos resultados mostraram que moscas com uma expressão diminuída ou aumentada destes genes, adoptam estratégias de defesa diferentes quando comparadas com os controlos. As primeiras *freezam* menos enquanto que as segundas *freezam* mais, o que sugere que a capacidade de aprender e memorizar características específicas do contexto é importante para a seleção dos comportamentos de defesa.

Esta descoberta suporta a hipótese de que a aprendizagem durante o período de exploração do ambiente onde o animal se encontra tem um papel importante na seleção de respostas de defesa e identifica 3 genes que estão, muito provavelmente, envolvidos neste processo, aumentanto assim o nosso conhecimento sobre como é que a familiaridade relativa ao contexto contribui para uma seleção adaptativa de estratégias defensivas.

Palavras-chave: *Drosophila melanogaster*, comportamentos de defesa, freezing, aprendizagem espacial

Abstract

The ability to perceive and respond to imminent threats is crucial to assure animal survival and species perpetuation. Remarkably, very little is known about how animals select particular defensive behaviors (e.g. freeze vs. flight) and how specific variables, such as spatial context familiarity, modulate these decisions.

To study how context familiarity affects the selection of defensive behaviors we exposed fruit flies to a looming stimulus – an expanding shadow mimicking a large object on collision course – in an inescapable environment. In such conditions, flies will typically run or freeze. Importantly, depending on the time they have to explore their spatial context, different strategies are adopted such that the longer the exploration, the more they freeze. Therefore, we hypothesized that the level of familiarity with the context may impact defensive behavior selection. To test this, we analyzed the defensive behaviors of *D. melanogaster* exposed to inescapable looming while manipulating the expression of memory and learning genes (*rutabaga*, *foraging* and *S6KII*).

Our results show that flies with reduced or increased expression of these genes, adopt different defensive strategies when compared to controls. The former freeze less while the latter freeze more, suggesting that the ability to learn and memorize specific context features is important for defensive behavior selection.

These finding lend further support to the hypothesis that learning during environment exploration plays an important role in the selection of defensive responses, and identifies 3 genes which are likely to be involved in that process, thereby increasing our understanding about how spatial environment familiarity contributes to the adaptive selection of defensive strategies.

Keywords: *Drosophila melanogaster*, defensive behaviors, freezing, spatial learning

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List of abbreviations

AC - Adenylate Cyclase

CA - Calyx

cAMP - cyclic AMP

cGMP - cyclic guanosine monophosphate

CS - Canton-S

CX - Central Complex

EB - Ellipsoid body

FB - Fan-Shaped Body

for - *foraging*

***for*$^{\mathbf{R}}$** - *for*$^{\text{Rover}}$

***for*$^{\mathbf{S}}$** - *for*$^{\text{Sitter}}$

GF - Giant fiber (GF)

LPLC2 - Lobula Plate/Lobula Columnar type 2

LC - Lobula columnar

MAPK - Mitogen-activated protein Kinase

MDNs - Moonwalker descending neurons

MBs - Mushroom Bodies

NO - Noduli

PED - Peduncle

PKA - Protein kinase A

PKG - cGMP-dependent Kinase

PB - Protocerebral bridge

RNAi - RNA interference

RSK - Ribosomal protein S6 Kinase

R neurons - Ring Neurons

rut - *rutabaga*

Introduction

The aptitude to perceive and escape imminent threats is crucial to assure animal survival and species perpetuation. Predation takes place in a highly dynamic and ever changing environment. Therefore, in order to be efficient, defensive systems have not only to be fast and robust, but also flexible. The timing and accuracy of the individual's response upon a threat are critical factors to assure the performance of an effective and optimized defensive behavior. This is critical considering that the cost of a slow and/or defective response is severe and might result in the death of the animal (Card, 2012). The need of mechanisms capable to allow these fast and accurate behaviors suggests that the neural circuitry responsible for these strategies relies on a small number of synapses, to diminish processing time (Card, 2012), and on large neurons (Sterling & Laughlin, 2015). Nevertheless, these circuitries should remain flexible enough so the individuals can adapt their response according the requirements of each particular context.

Upon an approaching predator, the animal will, most likely, perceive this object as a threat and will perform specific defensive behaviors in order to protect itself. In this situation, the individual will commonly choose to engage in one of three behaviors: freeze, flight or fight. Freezing behavior, is not a passive state of immobility (Fanselow, 1994), is a response characterized by complete immobilization and an increased attentional state towards changes in the surroundings, which helps to reduce the chances of an animal being noticed (D. C. Blanchard, Griebel, & Blanchard, 2001; Brandao, Zanoveli, Ruiz-Martinez, Oliveira, & Landeira-Fernandez, 2008; Egan et al., 2009; Fanselow, 1994; Zacarias, Namiki, Card, Vasconcelos, & Moita, 2018). Flight, or escape behavior, where the animal attempts to distance itself from the predator, can be described as a sequence of sub-behaviors. The nature of this set of sub-behaviors allows the escape program to be flexible and also allows the control of escape direction and reaction time (Card, 2012). Finally, fighting is also an option regarding defensive strategies. However, in a prey-predator interaction, engaging in a dispute with a predator might be very costly and inefficient for the animal and, therefore, it is not usually the favored strategy. Nevertheless, when the predator is already very close, the prey has been, most likely detected and therefore, fighting might be the only option since fleeing is not possible anymore. The choice is then between freezing and fleeing, in many cases. The defensive strategy adopted will, depend on the information gathered by the individual before and at the time of the threat detection. Before perceiving the threat, the animal is constantly gathering information about the surrounding space (e.g. availability of a refuge). This type of information will have an impact

on the chosen behavior upon exposure to a threat (de Oca, Minor, & Fanselow, 2007; Vale, Evans, & Branco, 2017). Moreover, when facing a threat, the animal will also evaluate characteristics of the dangerous agent (e.g. its speed and direction), and these cues will also have an impact on the final chosen behavior (De Franceschi, Vivattanasarn, Saleem, & Solomon, 2016; Tammero & Dickinson, 2002). Nevertheless, for threatening situations with exactly the same characteristics, the behavioral outcome might differ depending on circumstantial external and internal factors, such as hunger (Knobloch et al., 2012), specific individual differences (Eilam, 2005), the distance from the predator (Montgomerie & Weatherhead, 1988), the presence of offspring (Rickenbacher, Perry, Sullivan, & Moita, 2017), the presence of conspecifics (Ferreira & Moita, 2019), among others.

The "threat-approaching" condition can be easily mimicked in the lab using a virtual looming stimulus – a dark circle expanding in size at an exponential rate. The symmetrical expansion of this virtual expanding dot was suggested to be the stimulus feature that gives the individual information about the object's route and, therefore, the information that this object is on a collision course (Gibson et al., 2015; Lee, 1976). There is empirical evidence that support the fact that this visual pattern is indeed perceived as a real threat by a diverse range of animals, including humans (Ball & Tronick, 1971), non-human primates (Schiff, Caviness, & Gibson, 1962), rodents (Yilmaz & Meister, 2013), birds (Y. Wang & Frost, 1992), reptiles (Carlile, Peters, & Evans, 2006), fish (Temizer, Donovan, Baier, & Semmelhack, 2015) and invertebrates (Santer, Rind, Stafford, & Simmons, 2006), which all display defensive responses when exposed to looming stimuli.

Remarkably, very little is known about how animals select a particular defensive behavior, thus our major interest, in the present work, is to understand how specific external factors, such as the knowledge about the spatial context, can modulate these behaviors and contribute to this behavioral selection.

Drosophila melanogaster's defensive responses to looming stimuli

Looming stimuli have been demonstrated to induce a defensive reaction in *Drosophila melanogaster*. Card and Dickinson (2008b) demonstrated that, upon looming presentation, fruit flies can perform two different types of jumps in order to initiate flight: one of them is a stereotyped, but unprecise, fast escape, mediated by the giant fiber (GF); and the other is a slower but more precise response performed independently of the GF's activity. Card and

Dickinson (2008a) showed that *Drosophila* can use visual information provided by the loom to plan its escape behavior away from this threatening stimulus. Wu and Nern (2016) were interested in characterizing lobula columnar (LC) neurons, which are a type of visual projection neurons in *Drosophila*, that project to distinct central brain structures called optic glomeruli. Thus, after anatomically describing 22 LC types of neurons, they discovered that some of these cell types respond to looming stimuli while others are not activated by this kind of stimulation. Therefore, this study provided new knowledge about which cells might be responsible for the sensory input that ultimately produces looming-induced behaviors.

It is also known that the GF spike timing results from the summation of two visual features regarding the approaching object: its angular size and its angular velocity (Laurent & Gabbiani, 1998). Recently, it was discovered that the angular velocity encoding was attributed to the input of the LC type 4 (LC4) visual projection neurons and that the angular size component was provided to the GF by the Lobula Plate/Lobula Columnar type 2 (LPLC2) neurons (Ache et al., 2019). These authors also reported that both LC4 and LPLC2 neurons synapse directly onto the GF. A year later, Sen and colleagues (2017) discovered a specific population of visual projection neurons, that also respond to looming, the LC16 which activate the moonwalker descending neurons (MDNs) to trigger retreat in *Drosophila*. This retreat is done by backward walking (Bidaye, Machacek, Wu, & Dickson, 2014) which might allow the prey to elude the predator when trying to escape. Sen and colleagues (2017) conclude that LC16 and the MDNs are a crucial part of the neural circuit that transduces threatening visual stimuli into directed locomotor evasive output.

Gibson and colleagues (2015) asked what would be the behavioral response of fruit flies when exposed to a repetitive inescapable visual-threatening stimulus. In this case, the stimulus used was a shadow created by the translational movement of a rotary paddle that was fixed in above the walking arena. The authors reported that, in response to this stimulation, fruit flies exhibited elevated locomotor activity, presumably reflecting elevated arousal levels, mostly represented by increased walking velocity, as well as, repeated and persistent jumps. These authors also reported, for the first time, that, in response to the paddle movements, fruit flies displayed long periods of immobility similar to the freezing behavior previously described by Yilmaz and Meister (2013) displayed by rodents when presented with looming. Gibson and colleagues (2015) suggested that the behaviors displayed by the flies under this kind of stimulation, indicate an expression of a specific internal state, possibly comparable to a primitive emotion, analogous to what mammals experience as fear. In an independent effort,

Zacarias and colleagues (2018) invested in an extensive exploration and quantification of the behavioral responses and neural substrates underlying the responses of fruit flies' to inescapable looming. They observed that, when in a confined arena and under repetitive looming presentations, *Drosophila* most commonly either freeze or flee. Regarding the freezing behavior, Zacarias and colleagues (2018) showed that, in this inescapable repetitive looming paradigm, the fraction of fruit flies freezing increased gradually with each looming presentation. By the end of the stimulation period the majority of the flies were freezing. In their work and since running is known to be an alternative form of defensive behavior, these authors also analyzed the locomotor behavior of these flies (Gibson et al., 2015; Lebestky et al., 2009). In order to do that, they analyzed the flies that were not freezing during the stimulation period and observed that, during this period, walking speed increased relatively to the baseline period. Additionally, they also reported that flies sharply increased their speed upon each looming presentation. To evaluate if that was indicative of an escape attempt, they looked at the path orientations of the animals before and after each looming presentation. They discovered that, upon looming, there was a significant increase in the orientations towards the side of the chamber furthest away from the source of the threat (screen), which likely reflects escape attempts. Importantly, in this study, the authors found that the probability of performing either freezing or running depends on the fly's movement speed at the time of the loom presentation. This last finding strengthened the idea that there is an association between the decision of which behavior to perform and the individual's behavioral state at the time of threat perception. Finally, Zacarias and colleagues (2018) reported the discovery of a single pair of descending neurons (DNp09) that were shown to be essential for the performance of freezing behavior.

Both aforementioned studies reported escalating responses (i.e. increased walking speed and longer uninterrupted periods of freezing), probably due to the repetitive nature of the threat-like stimuli, suggesting that in this case, no habituation process was occurring. Importantly, all of these behaviors were reported to happen when fruit flies are facing natural predators (Parigi, Porter, Cermak, Pitchers, & Dworkin, 2014), which supports the ethological value of using virtual looming stimuli to study defensive behaviors in *Drosophila*.

Taking this knowledge into consideration, it becomes clear that the use of looming stimuli represents a very promising paradigm to investigate defensive behaviors and their neuronal underpinnings. However, and despite the advances afforded by the studies mentioned above, still very little is known about how the level of familiarity with the spatial environment impacts the selection of defensive behaviors in the fruit fly. Learning about the spatial features

of the environment is extremely useful for the animal to decide what to do upon a threatening situation, therefore, in the present study, we will take advantage of the previously mentioned paradigm to bridge this important gap in our knowledge. We aim to investigate in more depth which genes and neuronal circuits are involved in the acquisition, maintenance and update of previously acquired spatial information to guide the decision of what defensive behavior to engage upon a threatening situation. By combining the looming paradigm with an adequate animal model for which extensive and diverse experimental tools are available we can start addressing these questions in more detail.

Context familiarity

One of the most important factors regarding the spatial context of an animal is the presence or absence of a refuge and this aspect will modulate the defensive behaviors of the individuals upon a threatening situation (R. J. Blanchard, Flannelly, & Blanchard, 1986; Dill & Houtman, 1989; Vale, Evans, & Branco, 2018; Vale et al., 2017). The animal's surroundings have, indeed, been shown to be an important variable when it comes to decide what to do upon a threatening situation. It was reported that rodents, subjected to a threatening situation, when aware of the inexistence of a refuge in the surroundings, tend to preferentially freeze over trying to escape (de Oca et al., 2007; Vale et al., 2017) decreasing, this way, their changes of being noticed by the threatening agent (Eilam, 2005; Fanselow, 1994). Moreover, for instance, footshocks given immediately after rats are placed in a novel and closed environment trigger fleeing responses as opposed to the scenario in which they are given a brief exploration period before shock where freezing becomes the preferred behavior (Robert J. Blanchard, Fukunaga, & Blanchard, 1976). This exploration period seems to help the animal to get familiar with its inescapable context and adapt its behavioral choices.

Freezing has been previously described in fruit flies (Gibson et al., 2015; Zacarias et al., 2018), as mentioned above. It has also been reported that these animals can learn and recall spatial locations with remarkable efficacy (Foucaud, Burns, & Mery, 2010; Ofstad, Zuker, & Reiser, 2011). Furthermore, flies were shown to be able to learn in a fast and robust way, by trial and error, a certain unmarked location when an optogenetically delivered reward was available each time they visited that place (Stern et al., 2019), demonstrating, this way, the ability that flies have to learn a spatial task. As mentioned above, for mice, the exploration period a fly is given when in a new environment, will help it to get familiar with it and

understand whether or not there is a possible shelter or even an escape. It is possible that this gathered information could guide the individual's behavioral decisions when a threat is approaching. Thus, in order to choose the most adaptive behavioral response, an animal need to learn about their environment. Hence, we hypothesized that freezing would be the behavioral strategy most likely to be affected by differential levels of context familiarity.

When a fly is put inside an inescapable arena this place will be its new context. It will, therefore, explore it, displaying a high level of initial activity. This activity peak decays gradually with time which is thought to be a form of habituation to the arena through visual learning mechanisms (L. Liu, Davis, & Roman, 2007; Soibam et al., 2012). Eventually, it will learn whether there is an escape from this new place or not. Learning about the possibility of escape is of an enormous importance in case a threat is perceived. When presented with a repetitive looming stimulus flies after an exploration period in an enclosed arena will react according to their behavioral state (Zacarias et al., 2018) and possibly to what they know about their spatial context (Vale et al., 2017).

One way we have to manipulate context familiarity/knowledge about the spatial context is by changing the time the fly has to explore the environment before being exposed to looming stimuli, i.e. the duration of the baseline period. Ricardo Zacarias, a member from the Lab, performed this manipulation (see **Figure 1a**). The amount of information learned about the context will be different depending on the duration of the baseline. The longer the baseline, the longer the fly has to explore its spatial context, and, hence, the higher the likelihood of learning it is in an inescapable environment. These experiments indeed confirmed that baseline duration can impact the selection of defensive behaviors upon looming. These experiments showed that shorter periods of baseline resulted in fewer flies responding to looming with freezing and this was accompanied by a greater fraction of flies fleeing (see **Figure 1b,c**) (Zacarias, 2019), a result that was in agreement with our hypothesis above .

Moreover, results from a different experiment done in the Lab lend further support to the idea that the familiarity with the context play an important role in the selection of defensive behaviors. In this experiment a different behavioral paradigm was used, in which two enclosed arenas were used, instead of just one. These two arenas could be identical or have different shape, surface textures and visual patterns. In either case both arenas were connected by a small tunnel that allowed the flies to move from one arena to the other. During the baseline, the connection between the two arenas was closed, and the flies were only able to explore one of them. After the baseline period, the connection between the two arenas was opened allowing

the flies to move to the adjacent arena. This adjacent arena could be a copy of the first arena or a novel one. The goal with this new experimental design was to have two different environments which would open the possibility for an experiment where the fly would become familiar with one chamber but then receive looming stimulation in a novel and unexplored arena. Interestingly, flies which received looming in an arena identical to the one they explored during the baseline froze more than flies which were exposed to looming in a new arena that was different from the one explored during baseline, whose preferred response was running. Thus, this work showed that, when exposed to looming in a novel environment flies flee more than they freeze and, on the contrary, when threatened in a familiar arena, freezing becomes the preferred response. These findings suggest, once again, that context familiarity can indeed modulate the expression of defensive behaviors in *Drosophila*.

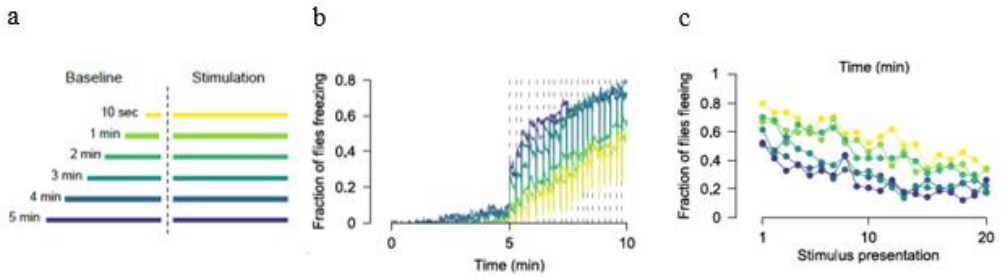

Figure 1. Influence of exploration time on freezing. Schematic representation of the different baseline durations tested (a), fraction of flies freezing for each baseline condition (dashed lines represent the looming stimuli) (b), fraction of flies escaping for each baseline condition during the stimulation period (each dot represents a looming stimulus) (c). Adapted from "Mechanisms of Defensive Action Selection in flies" by Zacarias, 2019

Besides being able to learn about the surrounding environment, animals need to memorize that information, and need to be able to retrieve it, should they encounter a threat. Thus, individuals with learning and memory impairments are expected to be compromised in acquiring context familiarity, and in particular in learning/memorizing the presence or absence of an escape. This led us to hypothesized that, in the context of our experiments, flies with learning and memory defects will tend to show comparatively lower levels of freezing, given their reduced ability to learn/memorize that there is no escape from the arena. We will test this hypothesis by subjecting flies with learning and memory deficits to repetitive looming stimuli under our behavioral paradigm, and quantify their defensive behaviors, prioritizing analysis regarding freezing.

Learning and Memory in *D. melanogaster*

As any other organism, fruit flies need to be able to navigate, perceive, learn and

memorize the environment in which they are, such that, they can use past experience to make informed and adaptive decisions throughout their lifetime. Memory and learning have been substantially studied in *D. melanogaster*. Several parts of the fruit fly's nervous system have been shown to be required for different types of learning and memory, namely, the Mushroom Bodies (MBs) and the Central Complex (CX) in the fruit fly's central brain.

The MBs are paired structures in the insect brain (Strausfeld, Hansen, Li, Gomez, & Ito, 1998; Technau & Heisenberg, 1982). Their neural network is mainly composed of Kenyon cells, dopamine neurons, output neurons, as well as other less numerous cells. The Kenyon cells are the most common cell type amounting to approximately 2500 in each side of this paired structure. The MBs can be divided into different anatomical and functional regions. Anatomically each of the paired of the MBs are made of a calyx, a peduncle and α, α', β, β' and γ lobes (Tanaka, Tanimoto, & Ito, 2008) (see **Figure 2**). Functionally they are divided according to the places to which the Kenyon cells project to. The MBs are known to be involved in a vast number of learning and memory processes such us: olfactory learning and memory (Davis, 2001; Heisenberg, Borst, Wagner, & Byers, 1985; Wolf et al., 1998), context generalization (L. Liu, Wolf, Ernst, & Heisenberg, 1999) and decision making (DasGupta, Ferreira, & Miesenbock, 2014; Tang & Guo, 2001; K. Zhang, Guo, Peng, Xi, & Guo, 2007).

The CX in *D. melanogaster* is composed of four major structures: the Ellipsoid body (EB), the Fan-Shaped Body (FB), the Noduli (NO) and the Protocerebral Bridge (PB) (see **Figure 2**) (Hanesch, Fischbach, & Heisenberg, 1989; Strausfeld, 1976). The EB, the most anterior substructure of the CX, is a perfectly round doughnut shaped substructure which can be divided in four concentric rings (Hanesch et al., 1989; Young & Armstrong, 2010). The neurons that make up the EB are called the Ring Neurons (R neurons). The FB is the largest component of the CX; It is composed of horizontal layers and vertical segments, and its neurons are referred to as F neurons (Hanesch et al., 1989; Renn et al., 1999). The PB is the most posterior part of the CX and can be separated into 16 segments. Finally, the NO are spherical structures located ventrally to the EB. The CX has been recognized as a center for controlling locomotor activities in arthropods (Homberg, 2008). Furthermore, this structure has been shown to be important for memory and learning functions in *D. melanogaster* and to act as a center for processing visual information and controlling visually-related learning behaviors. Vision provides the fly one of the most detailed pieces of information about the environment. Therefore, learning and memorizing visually acquired information becomes of major importance. Furthermore, it was recently found that flies have the ability to recall places based

on visual landmarks (Ofstad et al., 2011). Thus, being the CX a central brain structure recognized for its function related to vision, further investigation regarding its role is of major interest. Several studies have found the R neurons of the EB encode head direction (Seelig & Jayaraman, 2015) and are involved in a vast variety of visually-related learning behaviors (Guo et al., 2014; Neuser, Triphan, Mronz, Poeck, & Strauss, 2008; Ofstad et al., 2011; Pan et al., 2009). On the other hand, the F neurons innervating the FB were found to be specifically involved in visual pattern related learning. Together these findings suggest that different visual learning tasks are processed in different substructures of the CX. However, whether the CX substructures are directly involved in the acquisition, storage and retrieval of memory still needs more investigation.

In the fruit fly there are several genes that have been implicated in learning and memory. There are about 40 genes that have been linked to normal olfactory short-term memory and a subset of those have also been tested for their role in visual and place memory (Kahsai & Zars, 2011). Several studies investigated the function of these genes specifically in brain structures previously implicated in memory and learning (e.g. MBs and CX), and found they were required for a proper function of these cognitive functions (Levin et al., 1992; Putz, Bertolucci, Raabe, Zars, & Heisenberg, 2004; Z. Wang et al., 2008). In the present work we will investigate how the following genes contribute to the selection of defensive behaviors: *rutabaga*, *foraging* and *S6KII*. We chose these specific genes because they have been extensively studied regarding their role in memory and learning in *D. melanogaster* as will be explained in more detail next.

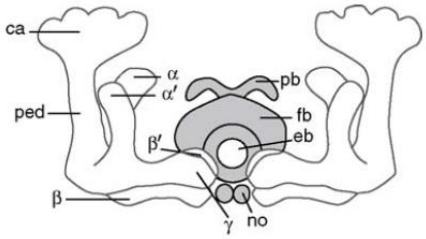

Figure 2. *Two neuropil structures, the MBs and CX, associated with learning am memory. MBs: calyx (ca), peduncle (ped), α, α', β, β' and γ lobes. CX: ellipsoid body (eb), fan-shaped body (fb), noduli (no) and protocerebral bridge (pb). Adapted from "Learning and memory in Drosophila: Behavior, genetics and neural systems" by Kahsai & Zars, 2011.*

The *rutabaga* (*rut*) gene encodes a calcium-calmodulin dependent Adenylate Cyclase (AC) (Dudai, Uzzan, & Zvi, 1983; Dudai & Zvi, 1985; Dudai, Zvi, & Segel, 1984; Livingstone, Sziber, & Quinn, 1984). This AC converts ATP into cyclic AMP (cAMP), one of the major signal transducers of the cell which is involved in a huge amount of cell responses to

environmental changes and plays a key role in plasticity. As mentioned above, the AC encoded by this gene is calcium-calmodulin dependent, meaning that for it to be active and properly functional it needs to bind to Ca^{2+} ions. The calmodulin protein binds to these ions and promotes their binding to the AC. Upon this neuronal activation, Ca^{2+} enters the cell through NMDA receptors (glutamate receptors) and binds to the AC which, will in turn convert ATP into cAMP leading to an increase of the intracellular levels of cAMP. The rising of this molecule will activate protein kinase A (PKA) which will initiate a phosphorylation cascade that ultimately will induce the expression of genes involved in learning (Davis, 1996; Fagnou & Tuchek, 1995). The expression of these genes will result in the establishment of new connections between neuronal cells while reinforcing preexisting ones, and such changes are basically the chemical and genetically driven basis of learning and memory processes. Thus, *rut* mutants, which will have lower levels of activity of the aforementioned AC, will have, most likely, their synaptic plasticity impaired, and thereby, will display learning and memory deficits. Zars (2000) showed that *rut* AC was needed exclusively in the Kenyon cells of the MBs for a component of olfactory short-term memory. Both Liu and colleagues (2006) and Pan and colleagues (2009), although some years apart, investigated where in the brain the proper expression of *rut* gene was necessary and sufficient for normal visual patter memory. The first authors identified this gene was needed in a subset of neurons from FB where a proper expression of *rut* was need in order to restore this type of visual memory. The latter authors evaluated similar features regarding visual pattern memory and discovered a subset of EB neurons that were also sufficient to rescue the visual pattern memory defect of *rut* mutants. With this work, it was possible to establish that *rut* gene was needed in FB for normal in visual pattern memory and, although not simultaneously, also in EB.

The *foraging* (*for*) gene encodes a cGMP-dependent Kinase (PKG) which is, as the name suggests, activated by cyclic guanosine monophosphate (cGMP) (Osborne et al., 1997). The cGMP-PKG signaling is of major importance for learning and memory (Kuntz, Poeck, & Strauss, 2017; Osborne et al., 1997; X. Wang & Robinson, 1997). The *for* gene is involved in food-search behavior in the fruit fly, and is an example of a single genetic polymorphism with two naturally occurring variants – *rover* and *sitter* – that ultimately result in two different feeding strategies (Sokolowski, 1980). for^{Rover} (for^{R}) flies travel longer path lengths during feeding and foraging behaviors than for^{Sitter} (for^{S}) flies (de Belle & Sokolowski, 1987). Regarding the biochemistry of these two variants, for^{R} is characterized by a higher relative PKG activity when compared to for^{S} (Osborne et al., 1997). The role of the *foraging* gene has been

intensively studied in *D. melanogaster*, and more recently, efforts were put together in order to better characterize its functions regarding behaviors other than food search. Therefore, studies investigating the role of this gene in learning and memory processes started emerging. The PKG encoded by the *for* gene has been shown to have functions related to neuronal excitability, synaptic transmission and neuronal connectivity (Renger, Yao, Sokolowski, & Wu, 1999) and to affect some types of non-associative learning (Scheiner, Sokolowski, & Erber, 2004), as well as olfactory associative learning (Kaun, Hendel, Gerber, & Sokolowski, 2007). Mery and colleagues (2007) discovered that for^{R} flies had higher short-term memory than for^{S} flies, reinforcing this way the impact of *for*-PKG activity on memory related processes. Wang and colleagues (2008) went a little further and demonstrated that for^{R} performed better than for^{S} flies in a visual pattern memory paradigm. They also found that to rescue this particular type of memory, in a foraging mutant background, a properly expressed *for* gene and therefore, higher levels of *for*-PKG activity were required in EB and FB. Additionally, they also showed that the rescue of this gene only on F5 neurons of the FB was sufficient to recover certain parameters of visual pattern memory. Regarding place learning it was later discovered that, contrary to olfactory and pattern memory, the difference of for^{R} and for^{S} PKG activity did not differently affect place learning (Gioia & Zars, 2009).

The *S6KII* gene of *D. melanogaster*, also referred to as *ignorant* gene (*ign*), encodes a ribosomal protein S6 Kinase (RSK) II (Wassarman, Solomon, & Rubin, 1994). This protein has been implicated in the Mitogen-activated protein Kinase (MAPK) signaling cascade in fruit flies (Kim, Lee, & Han, 2007). Putz and colleagues (2004) showed that this gene was required for operant place learning and pavlovian olfactory conditioning. Some years later, Neuser and colleagues (2008) reported that flies possessed spatial working memory, and found that the EB but not the MBs was necessary for this working memory. They ended up narrowing even more their study, and discovered that the *S6KII* gene is required in R3 and R4d neurons of the EB for normal working memory. More recently an interesting bridge between *S6KII* and *for* genes regarding memory and learning processes was brought to light. Kuntz, Poeck and Strauss (2017) showed that the *for*-PKG was required upstream of the S6KII protein in the ring neurons of the EB for proper visual orientation memory.

Goals of Present Work

The aim of the present work is to understand how learning about the spatial context can modulate the defensive responses of *D. melanogaster*. In order to achieve this goal, we are

taking advantage of the fruit fly's relatively tractable nervous system and of the arsenal of genetic tools available, to ask if the function of genes previously implicated in learning and memory processes, can impact the selection of defensive behaviors. Importantly, the large sample sizes afforded by this model system, allow us to obtain rather detailed quantifications of its defensive responses.

We investigated if alterations in the expression of specific genes, which are known to be involved in learning and memory processes in fruit flies, led to a change in the selection of defensive behaviors. We were especially interested in changes in the percentage of freezing, since this is the defensive behavior that is most likely to be affected by a learning/memory impairment which ultimately results in a lower level of context familiarity. We hypothesized that flies with this type of memory and learning deficits would display lower levels of freezing since their ability to perceive the inescapable properties of the environment would be compromised and their level of context familiarly would be reduced. Thus, we investigated the effects on the fruit flies' behavioral output of (1) reduced expression of these genes, (2) overexpression of these genes and (3) different exploration time durations on animals with genetic backgrounds with different doses of these genes. We tested this by performing experiments in which we used the GAL4/UAS system to either knockdown or overexpress the genes under study. We also used mutants of those same genes, which allowed us to evaluate the effect of having different numbers of gene copies, and, simultaneously how manipulations in baseline length impacted the selection of defensive behaviors. We were able to identify different genes that are simultaneously related to learning and memory functions, and that disrupt freezing behavior when not properly expressed. These results support our initial hypothesis that flies with memory and learning impairments have their ability to perceive the inescapable properties of the environment compromised and their level of context familiarity reduced which, ultimately, we believe leads to a higher tendency to escape and lower levels of freezing.

By exploring how defensive behaviors, are modulated by learning about the spatial context and, thereby, context familiarity, we hope to bring to light new knowledge about the mechanisms underlying the selection of defensive strategies of *Drosophila melanogaster*, and in particular how that selection is modulated by the external environment.

Methods

Fly husbandry

Stock maintenance

Flies were raised at 25ºC and 70% humidity in a 12h:12h dark:light cycle. Flies were kept in bottles if required at high numbers for experiments or in vials if required at low numbers, and fed on a standard Vienna medium (molasses, beetroot syrup, corn flour, granulated yeast, soy flour, agar, distilled water, propionic acid, nipagin and bavistina). They were flipped every 2 or 3 days to prevent overcrowding of the progeny and after 2 weeks the parental generation was replaced with younger flies.

Experimental flies

The flies used in behavioral experiments were 3-6 days old mated females. These flies were collected, after eclosion, under CO2, from the bottles where they were being raised and transferred to a vial with the same food medium. They were kept in these vials under the same conditions as rearing described above until the day of the experiments. These animals were kept in a 3:1 female:male ratio to ensure optimal mating, being the maximum density 21:7 to avoid overcrowding.

Fly Strains

The wild-type Canton-S (CS) strain (obtained from Ribeiro Lab) and the Nsyb-GAL4 (Bloomington (BL) # 39171) line had previously been acquired by the Lab. The following mutants and UAS-RNAi lines were obtained from the Bloomington Drosophila Stock Center: *rutabaga* mutant (rut^{2080}) (BL# 9405), $foraging^{Sitter}$ – (for^{S}) (BL# 76120), UAS-*EGFP*-RNAi (BL# 41553), UAS-*for*-RNAi (BL# 31698), UAS-luciferase(*luc*)-RNAi (BL# 31603), UAS-*rut*-RNAi (BL# 27035) and UAS-*S6KII*-RNAi (BL# 56031). Homozygous mutants were crossed with the CS line in order to obtain heterozygous flies, as will be showed in the results section of this work. The Nsyb-GAL4 driver line was crossed with the different UAS-RNAi lines in order to obtain offspring that had a pan-neuronal knockdown of those genes.

The UAS-*EGFP*-RNAi and UAS-*luc*-RNAi were used as controls in the knockdown experiments. The former was used as a control in the knockdown experiments with *S6KII* and the latter in the ones with *for* and *rut*. This choice was made based on the type vector that carries

the RNAi construct, and according to the landing site where that vector is inserted in the fly's genome.

Tools

GAL4/UAS System

The GAL4-UAS system is designed for targeted gene expression that allows spatial and/or temporal selective expression of genes of interest (Brand & Perrimon, 1993). This tool constraints the expression of a certain gene to specific cells where both the transcription activator protein, encoded by the GAL4 gene (GAL4), and the UAS sequence are present. After being translated, GAL4 protein binds to the UAS sequences in the cells where both these elements are present. By binding to the UAS enhancer sequences, GAL4 promotes the expression of a desired gene of interest, in those cells (see **Figure 3**). This way this system can work as a switch to gene expression depending on the presence or absence of these elements.

In order to get these two parts of the system together we need to cross a fly line that expresses GAL4 with a line containing the UAS sequence upstream of the gene we wish to ectopically express (see **Figure 4**). The UAS enhancer sequences will be present in every cell but the GAL4 protein will only be present in a specific set of cells, the ones in which the promoter that controls GAL4 expression is active. This way, by crossing a GAL4 line with a UAS line, we will be expressing the GAL4 protein only in specific tissues of interest and, therefore, this transcription activator protein will only bind to the UAS sequence in these specific places where both UAS and GAL4 are present. Thus, the transgene of interest, will only be expressed, and ultimately have an effect, in those regions.

In our experiments we used this system with Nsyb-GAL4 in which GAL4 expression is under the control of a pan-neuronal promoter (Nsyb), and thereby allows broad expression in all of the neuronal cells (Lin & Goodman, 1994). Regarding the UAS lines we worked with UAS-RNAi lines. RNA interference (RNAi) disrupts gene activity by reducing the levels of mRNA that are expressed (Dietzl et al., 2007). By using the Gal4-UAS system to express different RNAi molecules we were able to disrupt the expression of our genes of interest and analyze the behavioral output of these manipulations.

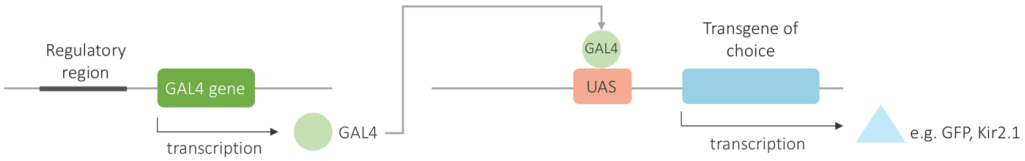

Figure 3. GAL4-UAS system. The transcription of the GAL4 gene results in a GAL4 protein which will drive the expression of a transgene of interested once it binds to a UAS regulatory region.

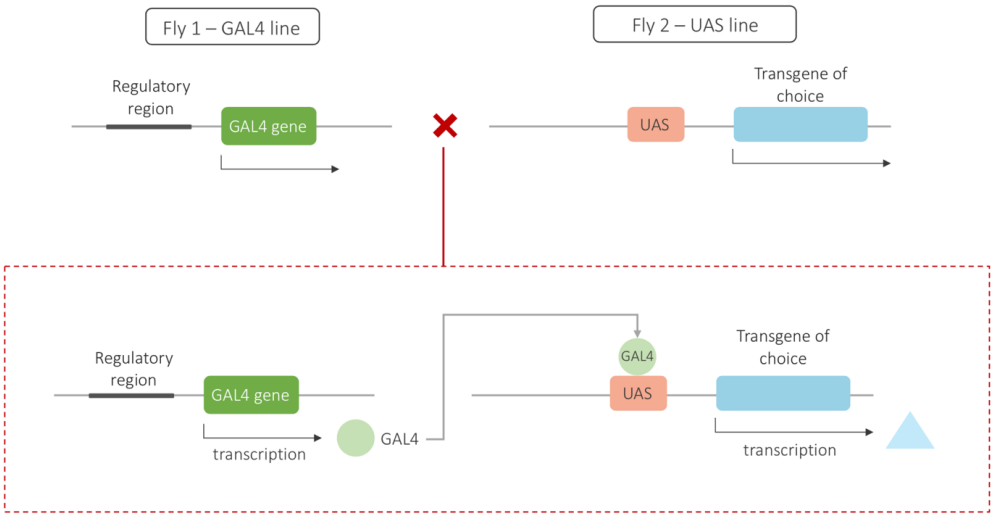

Figure 4. Crossing a GAL4 line with a UAS line in order to restrict the expression of the transgene of interest to a set of specific neurons.

Behavioral Apparatus

We recorded behavior of single flies in response to inescapable looming stimuli in a custom-built setup which is housed in an experimental room with the same temperature, humidity and light cycle conditions as those used for rearing (see above). The setup is enclosed in an opaque black box to decrease exposure both to light and to the experimenters (see **Figure 5a,b**).

We tested four flies per trial in a stage built to hold four custom-built arenas with the following dimensions: 30 mm in diameter and 3 mm in height (since the lid has 1 mm, the arena measures 4 mm in height when covered by with the lid) (see **Figure 5c**). The walls of these arenas were made of white opaque acrylic, such that flies tested simultaneously could not see

each other, and were covered by a transparent acrylic panel which prevented the flies from escaping. Single flies were aspirated into an arena, which were then placed on the stage.

Between the stage where the arenas are placed and a custom-built LED array placed underneath that stage, there was a 2 mm white opaque acrylic sheet to diffuse the light that came from the LED, in order to obtain a homogeneous illumination of the arenas (see **Figure 5b**). The infrared LED array retro illuminates the arenas serving, therefore, as a backlight for locomotion imaging.

The screen where the stimulation was presented was placed 17.5 cm away from the arenas at a 45º angle. To record the experiments, a high-speed camera was placed above the set of four arenas that recorded the flies at 60 frames per second (fps). This camera had an 850 nm pass filter (visible light filter) to reduce visual noise.

An infrared LED connected to an Arduino circuit was set in the center of the 4 arenas. This LED blinked when each trial started and ended, and every time there was a looming stimulus (see **Figure 5d,e**). This way, the alignment of the start of the videos as well as the onset of each loom during the analysis were facilitated.

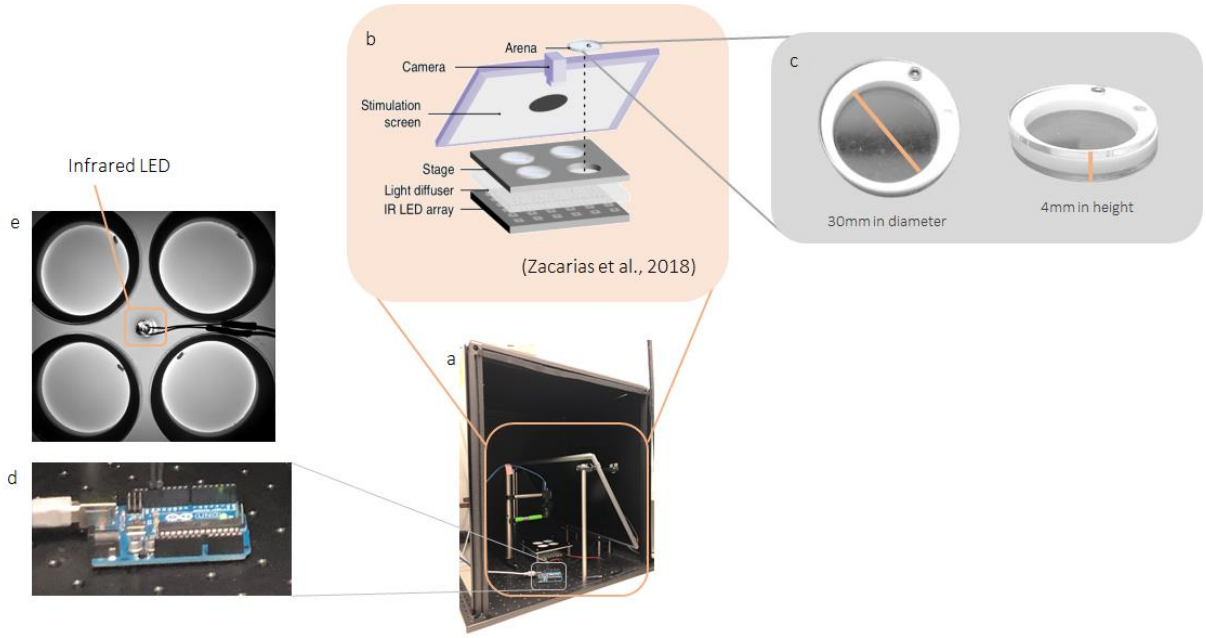

Figure 5. Behavioral Apparatus. Experimental setup (a), scheme of the experimental setup (b), arenas (c), arduino circuit (d) and example of a frame from an experimental video showing the central infrared LED (e).

Visual Stimulation

The stimuli were delivered by a 24-inch monitor (ASUS VG248QE) running at 144Hz. To generate looming effect, a 100% solid black circle increased in size over a white background (see **Figure 6**).

The visual angle of the expanding circle, at each frame, was determined by the equation: $\theta(t) = 2\tan^{-1}(l / vt)$, where $\underline{l}$ is half of the length of the object, $\underline{v}$ the speed of the object towards the fly (cm/s), and $\underline{t}$ is the time to collision (seconds). In our experiment, we set $l = 1$ and $v = 25$, to simulate the visual dynamics of an object with 1 cm radius that is approaching at a constant velocity of 25 cm/s.

Each looming presentation lasted for 500ms. The looming stimuli appeared on the screen 500 ms before collision ($\theta = 9°$), and expanded during 450 ms until it reached the maximum size of 78º where it remained for 50 ms before disappearing. The final size reflected the largest circle that could still fit inside the monitor.

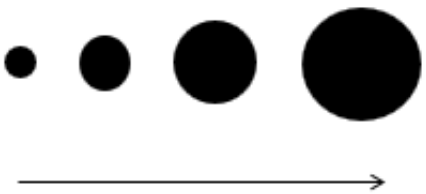

Figure 6. Visual stimuli. Expanding black solid circle that generates the looming effect.

As the circle expands, a considerable decrease in luminance within the behavioral apparatus occurs. When no stimulus is being presented (white screen) the luminance at the stage where the behavioral arenas are places is 260 lux, and just before looming offset, when the disk reached its maximum size, the luminance is 32 lux, representing a 88% decrease in luminance.

All behavioral protocols were generated in custom Python scripts using PsychoPy (Peirce, 2007). All protocols used included a baseline period, to allow the flies to recover from the aspiration step, which could vary (30 seconds, 1 minute or 5 minutes) and during which the screen was kept white. This baseline period was followed by a stimulation period of 5 minutes where the flies would be exposed to 20 looming stimuli distributed in a pseudo-randomly manner with an inter-stimulus interval between 10 and 20 seconds (see **Figure 7**).

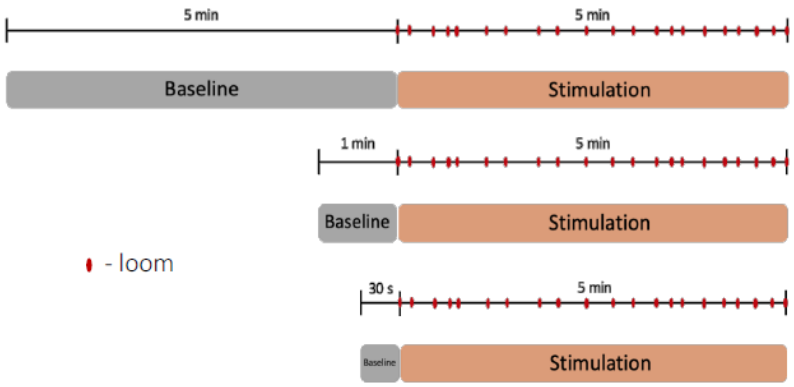

Figure 7. Scheme of the different behavioral protocols used.

Visual acquisitions, fly detection and tracking

The videos of the experiments were acquired using Bonsai (Lopes et al., 2015) at 60Hz and 1140x1040 resolution. Image segmentation was then performed by custom software in Python using OpenCV. The main features extracted from the videos were the fly position and the motion activity around the fly. The position was calculated from the centroid of an ellipse fitted to the fly by background subtraction; the motion was quantified by the number of pixels active in a 100x100 pixel region of interest surrounding the fly (a pixel was considered to be active if it recorded a change higher than 10 intensity levels) (see **Figure 8**). The fly position was then used to compute the fly's velocity, and the motion was used to calculate freezing and distinguish it from slow movements, such as grooming.

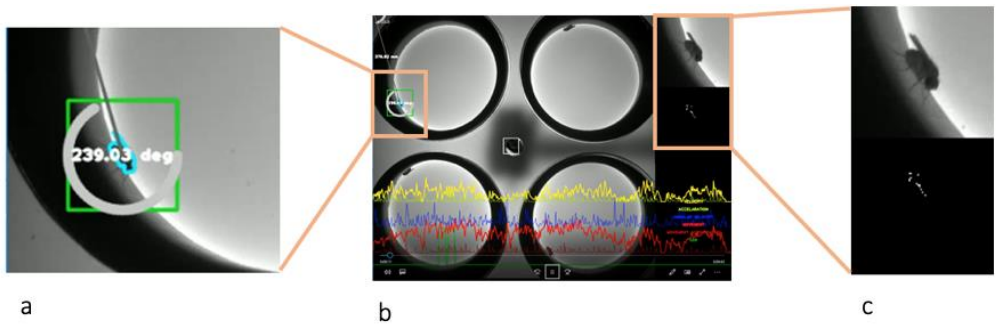

Figure 8. Video analysis. Computation of the fly position (a), output video of the tracking software showing acquired variables (b) and computation of fly motion (c).

Behavioral classifiers for behavioral analysis

Pre-established behavioral classifiers were used in order to automatically classify behavioral states, speed and motion. The thresholds values used for the classifiers were set by Zacarias and colleagues (2018) through manual annotation of the flies' behavior during trials. The tracking data was averaged into 500ms bins in order to facilitate the classification.

Walking: A fly was considered to be walking if its average walking speed exceeded 4mm/s (see **Figure 9b** - green).

Grooming: Low speed behaviors performed while not walking. A fly was considered to be grooming if it exhibited speed lower and 4mm/s and an average pixel change around itself higher than 50 pixels/s (see **Figure 9a,b** - blue). A minimum change of 10 intensity levels from one frame to the next was required for a single pixel to be considered active.

Freezing: A fly was considered to be freezing when the average motion (pixel change) around the fly was lower than 50 pixels/s (~5% of the fly area) and its speed lower than 4mm/s (see **Figure 9a,b** – red). We used pixel activity/motion to classify freezing bouts because, as mentioned above, flies can exhibit low speed behaviors while not walking (grooming). Since freezing is a sustained response that can last for several looming presentations, we consider a freezing response to a given looming presentation even if the fly initiated freezing upon a prior looming stimulus. Considering that a fly can be already freezing upon a looming presentation, it could be difficult to assert whether it is responding to that stimulus or not. Nevertheless, we observed that flies freezing display startles at the time of the looming. These startles are, therefore, a strong indicator that the animals already freezing are still responding to the stimulus. It is important to note that we did not include the analyze regarding the levels of freezing during the baseline period of our experiments. In most cases, there were significantly differences detected relatively to this variable in this period, but the overall levels of freezing during this initial time were globally very low. Furthermore, the differences between conditions although statistically significant were also very low. Therefore, this analysis will not be present in the present work.

Jumps: A fly was classified as having jumped if its instantaneous speed exceeded 75mm/s, a threshold identified by a discontinuity in speed distribution.

Escapes: An escape response was considered every time one of the following events was verified: the fly jumped upon the looming presentation; upon looming presentation, the fly turned away from the screen, or if it was already facing away from the screen, it kept a walking trajectory towards the side furthest away from the source of the stimulus; the fly increased its walking after the looming presentation independently of the trajectory taken.

Under the scope of the present work, we focused on freezing behavior, walking speed and escapes.

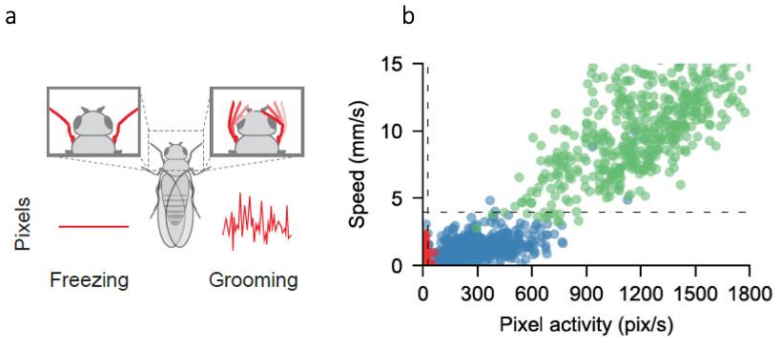

Figure 9. Behavioral classifiers. Scheme of the pixel activity during freezing and grooming (a). Correlation between pixel activity and speed: red - freezing, blue - grooming, green – walking (b). Adapted from "Speed dependent descending control of freezing behavior in Drosophila melanogaster" by Zacarias et al., 2018, *Nature communications, 9(1), 3697*

Statistical Analysis

The data analysis is performed using custom Python scripts and prior to all statistical testing the data is tested for normality with Shapiro-Wilk normality test, and the appropriate non-parametric test is chosen if the data is not normally distributed. All statistical tests are two-sided, and all statistical tests used are specified in the results section. To quantify group differences, we used the Kruskal-Wallis test since the behaviors under analysis never followed a normal distribution. When analyzing more than two groups at the same time we used the Dunn post-hoc test, in addition to the Kruskal-Wallis test. We used the z-test for independent proportions to evaluate if there were significant differences between the final proportion of flies freezing for the conditions under study. All the tests performed belonged to the SciPy python library and all the plots were acquired using the Python Matplotlib library.

Results

The experiments reported in this section were planned, executed and analyzed in close collaboration with Ricardo Neto Silva, a post-doc in the Lab. In the first part of this section, I quantify looming triggered defensive behaviors displayed by fruit flies in which we knocked down the *rutabaga*, *foraging* and *S6KII* genes, independently. Secondly, I present the same type of analysis regarding flies in which we overexpressed the *rutabaga* gene. Finally, I quantify and describe the behavior observed in mutant flies for the *rutabaga* and *foraging* genes. In this last section, I analyze the effects of different gene doses in combination with different baseline durations on the behaviors under analysis. Our main goal was to identify phenotypes related to freezing behavior, since, as mentioned before, this is the behavior most likely to be affected by a learning or memory impairment.

I opted to include here only the results in which statistically significant differences were detected to make the text lighter and straight forward. However, all the non-significant quantifications are presented in the Annexes of the present work and will be properly identified throughout the following descriptive text.

Effects of *rut, for* and *S6KII* knockdowns on *D. melanogaster* defensive behaviors

In the following experiments, the expression of the RNAi transgenes was driven by a Nsyb-GAL4 driver line. This pan-neuronal driver allowed us to knock down the target genes in all neuronal cells, without affecting other tissues although several RNAi lines are available. We chose to work with RNAi lines from the TRiP collection, for the large number of lines available and the possibility to use RNAi lines for non-endogenous fly genes as controls. For *S6KII* and *for* there are several different lines available in the TRiP collection. Although we tested different lines available for each of those genes, here we will only show the results for the RNAi lines that showed the biggest effect on freezing.

rut knockdown

Figure 10 depicts the behavior of the *rut* knockdown flies. During the stimulation period, relatively lower levels of freezing are observed for the *rut*-knockdown flies, which is reflected both by a lower fraction of flies freezing (29/75) compared to control flies (49/79) (z-test for independent proportions, $p < 0.01$, see **Figure 10a**), and also by a reduced percentage of time spent freezing during stimulation (control: median = 46.3333%, IQR = [4.0833; 81.0];

rut-RNAi: median = 3.8333%; IQR = [0.0; 55.3333]. Kruskal-Wallis test, $p < 0.001$, see **Figure 10b**).

Given the reduced levels of freezing observed for flies with neuronal knockdown of *rut*, we next analyzed if and how other defensive behaviors were affected. To look at escapes, we started by analyzing their locomotor activity excluding all freezing and grooming bouts, hence only periods classified as walking (see **Figure 10c**). During baseline, *rut*-knockdowns displayed lower speeds compared to control flies (control: median = 8.2534 mm/s, IQR = [7.1544; 10.0469]; *rut*-RNAi: median = 7.2202 mm/s, IQR = [6.3419; 8.0949]. Kruskal Wallis test, $p < 0.001$, see **Figure 10d**), and while we observed that both conditions increased their speed upon stimulation, the walking speed of the *rut*-RNAi flies was still lower than controls (control: median = 11.1698 mm/s, IQR = [9.7178; 13.0115]; *rut*-RNAi: median = 10.1933 mm/s, IQR = [8.4324; 11.8682]. Kruskal-Wallis test, $p < 0.05$, see **Figure 10e**). Since Zacarias and colleagues (2018) had previously found that freezing probability is lower for higher walking speeds just before looming onset, we analyzed the walking speed in the 1 second period that precedes each looming, and found that it was significantly lower for *rut*-RNAi flies (control: median = 10.8944 mm/s , IQR = [9.3101; 12.2857]; *rut*-RNAi: median = 9.3662 mm/s, IQR = [8.1793; 10.9453]. Kruskal-Wallis test, $p < 0.05$, see **Figure 10f**). Regarding this last observation, because these impaired flies are displaying lower walking speeds and even though freezing less than controls, suggests that their low levels of freezing behavior are not expected and support our hypothesis that this pattern might be due to their inability to learn the context.

To further analyze the increase in walking speed during the stimulation period, we focused on changes in speed around the looming stimuli, by quantifying the difference in walking speed between 30 frames after looming and the same period before looming. We found that, although both conditions increased their speed after the looming, this change was significantly lower for the *rut*-RNAi flies (control: median = 3.8680 mm/s, IQR = [2.0384; 6.8249]; *rut*-RNAi: median = 2.7695 mm/s, IQR = [1.2835; 4.3367]. Kruskal-Wallis test, $p < 0.05$, see **Figure 10g**). This suggests that, although these flies seem to react to looming stimulus, they might be being display less vigorous escape attempts compared to controls.

We next asked if the running responses observed corresponded to escape attempts away from the threat. We analyzed the orientations of the walking paths relatively to the screen where the stimuli were being presented, before and after each looming and found that for both conditions, flies changed their walking orientation upon looming away from the screen. The

difference in walking orientations, before and after the looming, was indeed significantly different for both control and *rut*-RNAi flies (Mann-Whitney test, $p < 0.001$, see **Figure 10h**).

Our hypothesis predicts that compromised context familiarity, should be manifested in reduced freezing, and higher number of attempted escapes. Therefore, we compared the number of flies escaping between *rut*-knockdown and control conditions during the stimulation period, and we found, that the reduced freezing is indeed accompanied by a higher fraction flies escaping for *rut*-knockdown flies (see **Figure 10i**) almost the entire time. This analysis was only qualitative, however we plan to further investigate whether the apparent differences are statistically significant.

Finally, we compared fraction of flies freezing, fraction of flies escaping and fraction of non-responder flies for controls (see **Figure 10j**) and *rut*-RNAi (see **Figure 10k**) flies during the stimulation period. Upon the first loomings, the majority of the flies displayed escape responses, and the freezing fraction was reduced. As the stimulation continued, we observed an increase in freezing and a decrease in escape attempts, such that after a certain time point the freezing fraction became higher than the escapes fraction. However, the increase in freezing and accompanying reduction in escapes, occurred faster for control flies than for *rut* knockdown flies. Regarding the fraction of non-responder flies, we observed that this value seems to be higher for the *rut*-knockdown flies than for controls. Again, this analysis was merely qualitative and further statistical analysis are in our future plans.

All the quantifications regarding this experiment that were not statistically significant are shown in **Annex A**.

In summary, these results show that flies with lower neuronal levels of *rut* display lower levels of freezing under the conditions of our paradigm and although they display more in escape attempts, they seem to do it in a less vigorous way compared to controls. Moreover, these impaired flies tend to display overall lower walking speed levels compared to controls.

for knockdown

Figure 11 depicts the behavior of *for* knockdown flies. During stimulation, *for*-knockdown flies froze less than controls, which is manifested by a lower fraction of flies freezing (25/65) compared to controls (48/66) (z-test for independent proportions, $p < 0.001$, see **Figure 11a**) and also by a lower percentage of time spent freezing during that same period (control: median = 77.25%, IQR = [32.83; 92.08]; for-RNAi: median = 0.5 %; IQR = [0.0; 50.1667]. Kruskal-Wallis test, $p < 0.001$, see **Figure 11b**).

Figure 11c shows the walking speed of these flies during the experimental time. *for*-RNAi flies displayed higher walking speed values during the stimulation period (control: median = 12.73 mm/s, IQR = [10.64; 14.69]; *for*-RNAi flies: median = 14.2996 mm/s, IQR =

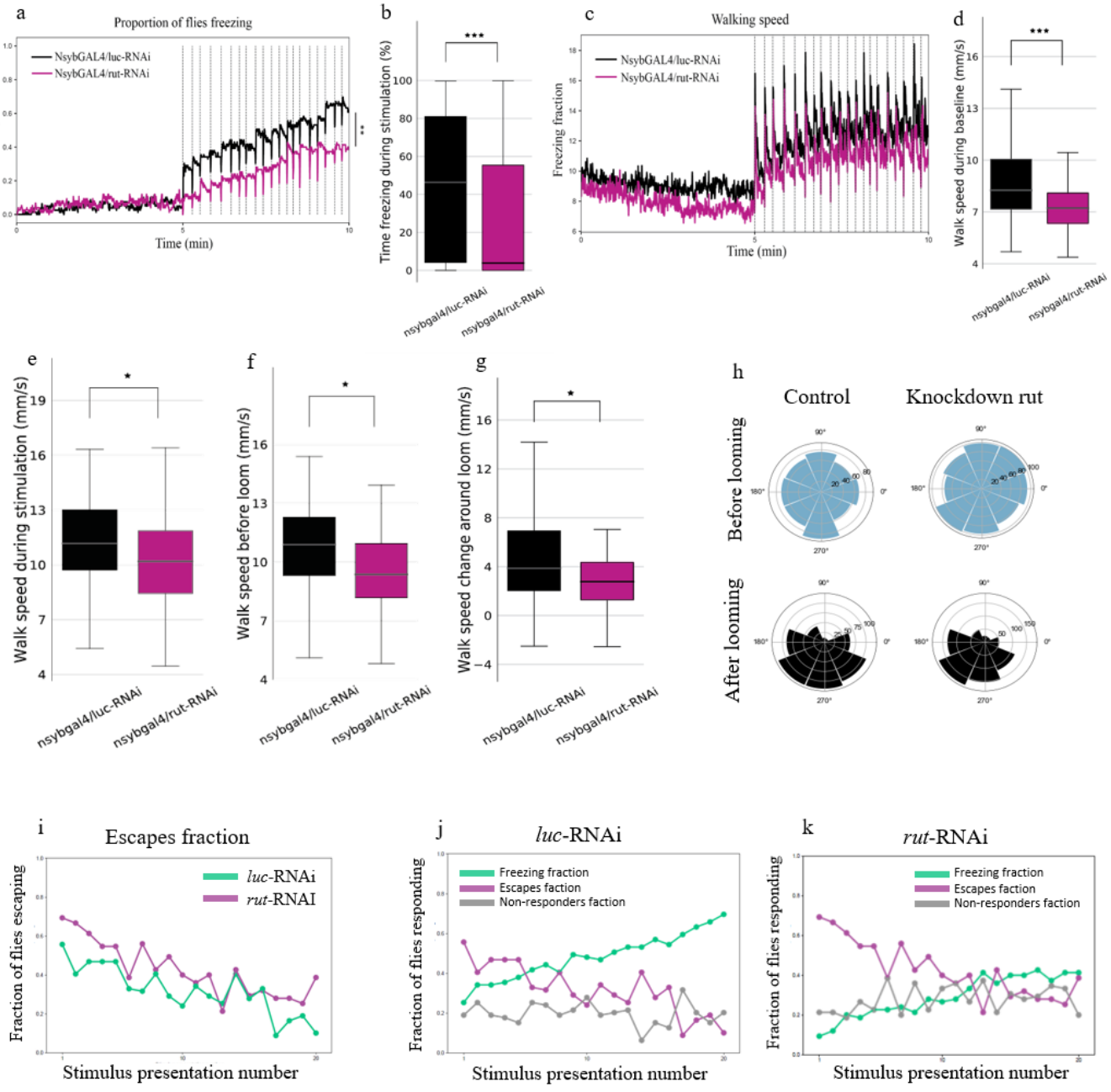

Figure 10. Behavioral analysis of *rut*-knockdown flies. Proportion of flies freezing. Dashed lines indicate stimulus presentations (a), percentage of time spent freezing during the stimulation period (b), average walking speed across the experiment (c), walking speed during the baseline period (d), walking speed during the stimulation period (e), walking speed before the looming presentation (f), change in walking speed caused by stimulus presentation (pre-stimulus period subtracted from post-stimulus period) (g), distribution of path orientations for both control and *rut*-knockdown flies before and after looming walking trials. Bar height indicate counts (h), fraction of flies fleeing for each looming presentation (i), proportion of flies performing each of the described behaviors (j) and (k). * denotes p<0.05; ** denotes p<0.01; *** denotes p<0.01. Sample sizes: control n =79, *rut*-knockdown n =76.

[12.43; 15.75]. Kruskal-Wallis test, $p < 0.01$, see **Figure 11d**), which is in contrast to what we found for *rut* knockdown flies. Moreover, the difference in walking speed between stimulation and baseline periods was higher for the flies with reduced neuronal levels of *for* (control: median = 2.146 mm/s, IQR = [0.5039; 3.4789]; *for*-RNAi flies: median = 2.95 mm/s, IQR = [1.93; 3.865]. Kruskal Wallis test, $p < 0.05$, see **Figure 11e**). **Figure 11f** shows that the speed before the looming was significantly higher for *for*-RNAi flies (control: median = 11.25 mm/s, IQR = [9.88; 13.06]; *for*-RNAi: median = 13.581 mm/s, IQR = [11.72; 15.224]. Kruskal-Wallis test, $p < 0.001$), which is the opposite to what we found for *rut* knockdown flies. As mentioned before, the higher the walking speed of flies just prior to the stimulus, the lower the probability that it will freeze. Therefore, these last observations would suggest that these flies were less prompted to freeze as a response to looming, therefore to distinguish the contribution of walking speed from the learning and memory impairment to the reduced freezing observed further experiments would be necessary which will be described in more detail in the Discussion section. Regarding the change of speed around looming, we observed that the increase in speed was significantly lower for *for*-RNAi flies (control: median = 2.5616 mm/s, IQR = [0.0652; 4.724]; *for*-RNAi: median = 1.4456 mm/s, IQR = [-0.1753; 2.2882]. Kruskal-Wallis test, $p < 0.05$, see **Figure 11g**), similar to what we described above for *rut*-RNAi flies. Nonetheless, *for*-RNAi flies were already displaying higher walking speed values compared to controls throughout the whole stimulation period, therefore, in this case, that might justify this lower increase in speeds upon stimulation (because they were already walking faster) and not necessarily that they are displaying less vigorous responses to looming. Both *for*-RNAi and control flies displayed significant changes in walking path orientations in response to loomings. (Mann-Whitney test, $p < 0.001$, see **Figure 11h**).

As for the *rut*-knockdown, the fraction of flies escaping is higher for the *for*-RNAi condition than for controls (see **Figure 11i**), which is again in agreement with our hypothesis related to context familiarity and selection of defensive responses since the fact that they might not be able to learn the absence of an escape leads them to keep trying to engage in that behavior, even though is not the most adaptive one in their particular context. Another explanation for this pattern regarding escapes, might be due to the fact that these flies had overall higher walking speeds.

Finally, **Figures 11j.k** show that in the beginning of the stimulation period, the escapes fraction is high for both conditions. However, as more looming were presented, we observed an increase in the freezing fraction and a reduction in the number of escapes. Importantly, and

as previously described for *rut* knockdowns, the increase in the freezing fraction in relation to the decrease in the escapes fraction was slower for the flies with impaired learning. Regarding the fraction of non-responder flies, we observed that this value is higher for the for-knockdown

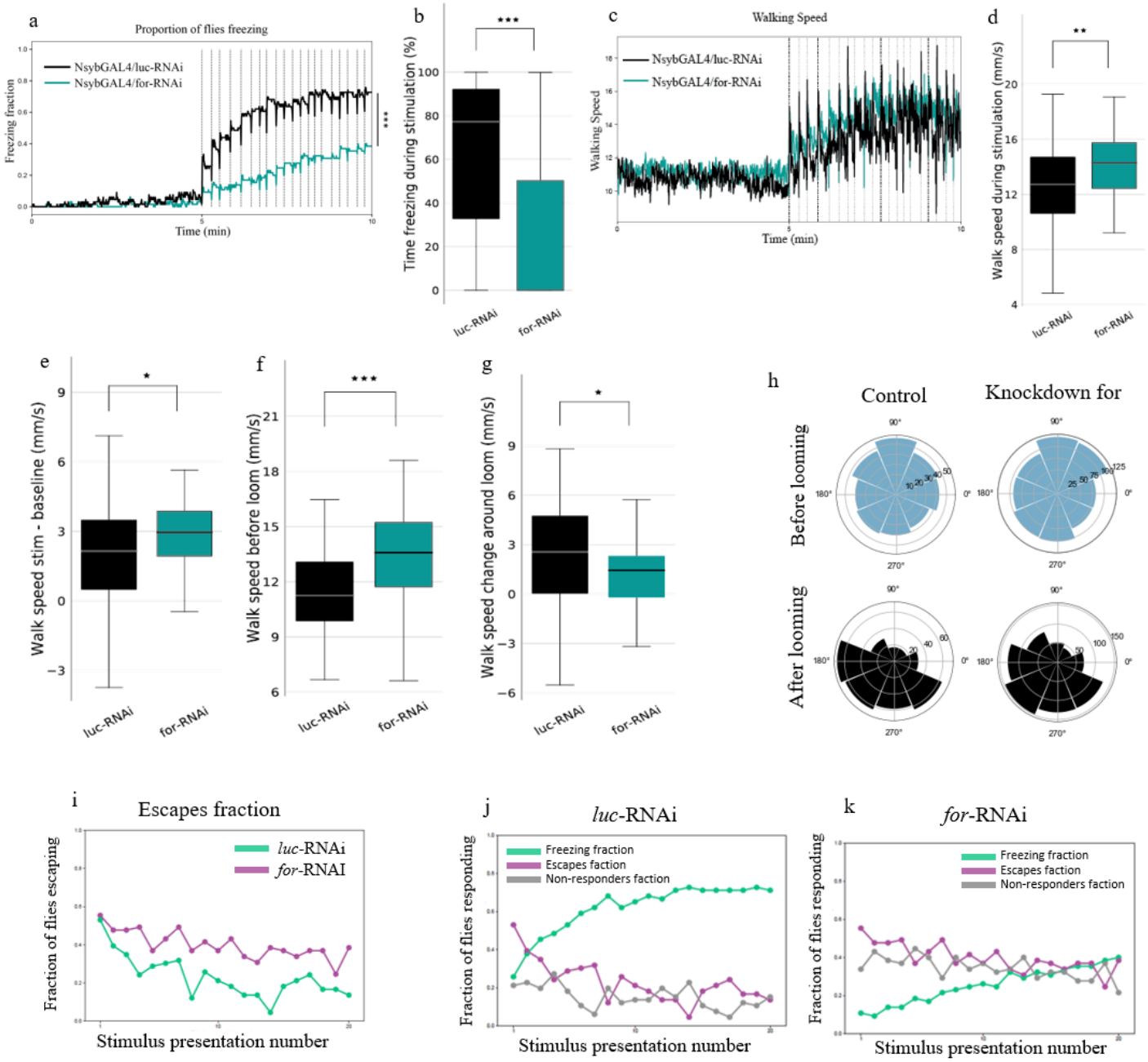

Figure 11. Behavioral analysis of *for*-knockdown flies. Proportion of flies freezing. Dashed lines indicate stimulus presentations (a), percentage of time spent freezing during the stimulation period (b), average walking speed across the experiment (c), walking speed during stimulation period (d), change in walking speed caused by the looming presentation (baseline period subtracted from stimulation period)(e), walking speed before the looming presentation (f) change in walking speed caused by stimulus presentation (pre-stimulus period subtraced from post-stimus period) (g), distribution of path orientations for both control and for-knockdown flies before and after looming walking trials. Bar height indicate counts (h), fraction of flies fleeing for each looming presentation (i),

proportion of flies performing each of the described behaviors (j) and (k). * denotes p<0.05; ** denotes p<0.01; *** denotes p<0.01. Sample sizes: control n = 66, *for*-Kncokdown n = 65.

flies than for controls and it is very similar to the fraction of flies escaping. This might be happening due to the threshold that we established for the change of speed in response to looming. Since these flies were already showing high walking speeds, the increase the display upon the looming might not be robust enough to be considered an escape attempt according to our classifiers. We need to revise these thresholds to see if this might be the cause of the high fraction of flies non-responding. Moreover, it is still possible that there are indeed more flies non-responding because of other possible impairments and in that case further experiments would be needed to disambiguate this option.

In summary, these results show that flies with lower neuronal levels of *for* display lower levels of freezing under the conditions of our paradigm which support our hypothesis that defective learning modulated the defensive behavioral choices of these animal. Contrary to what observed for *rut*-RNAi flies, *for*-RNAi ones displayed overall higher values of walking speed compared to controls which, because of the reasons described above might be the reason these flies are less prompted to freeze upon stimulation.

S6KII **knockdown**

Figure 12 depicts the behavior of the *S6KII*-knockdown flies. For the first few looming stimuli, and in contrast to the knockdown experiments described above, there were no substantial differences in the fraction of flies freezing between conditions (see **figure 12a**). Such differences only became apparent around the 5^{th} loom, and by the end of the experiment there were, indeed, significantly less *S6KII*-RNAi flies freezing (34/63) compared to control (47/64) (z-test for independent proportions, $p < 0.05$). The percentage of time spent freezing during stimulation was also lower for the *S6KII*-RNAi flies (control: median = 63.58%, IQR = [3.79; 89.79]; *S6KII*-RNAi: median = 18.0%; IQR = [0.1667; 79.3333]. Kruskal-Wallis test, $p < 0.05$, see **Figure 12b**).

Figure 12c depicts the walking speed of both test and control flies throughout the experimental time. During baseline, *S6KII*-RNAi flies displayed a significantly higher walking speed (control: median = 10.8192 mm/s, IQR = [8.957; 11.822]; *S6KII*-RNAi: median = 11.478 mm/s, IQR = [9.93; 12.629]. Kruskal-Wallis test, $p < 0.05$, see **Figure 12d**). However, in response during the stimulation period, we did not find any differences in our analysis of

walking speed between knockdown and control flies, suggesting that *S6KII* knockdown affects freezing specifically, without affecting escape speed (**Annex B**). Furthermore, both conditions changed their orientation away from the screen after the looming (control: Mann-Whitney test, $p < 0.001$; *S6KII*-RNAi: Mann-Whitney test, $p< 0.001$, see **Figure 12e**).

Figure 12f shows that during stimulation the fraction of flies escaping was higher for *S6KII* knockdown flies throughout almost the entire time, and in particular for the second half of the stimulation period, which is consistent with the freezing fraction pattern observed in **Figure 12a**.

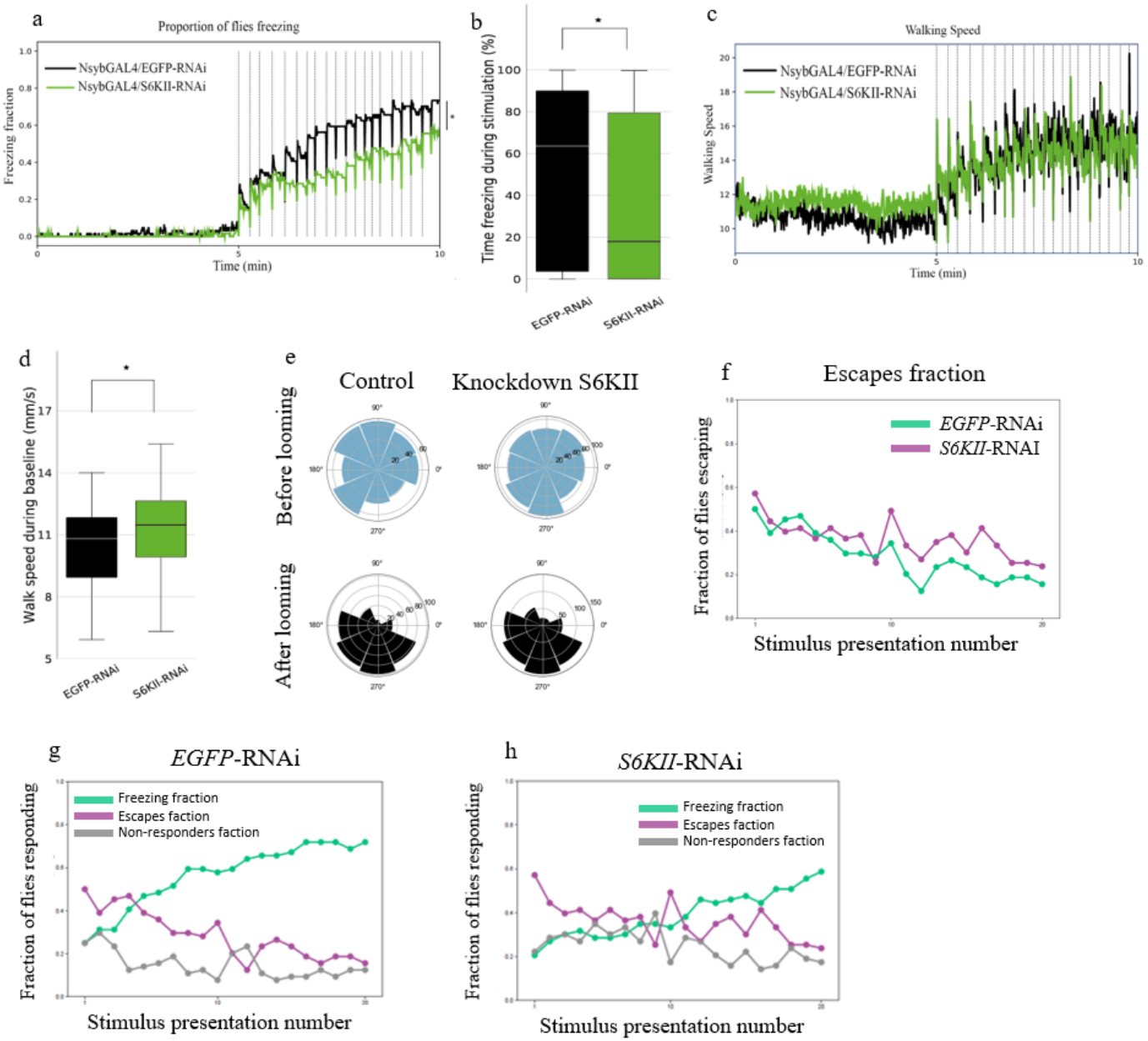

Figure 12. Behavioral analysis of S6KII-knockdown flies. Proportion of flies freezing. Dashed lines indicate stimulus presentations (a), percentage of time spent freezing during the stimulation period (b), average walking

speed across the experiment (c), walking speed during baseline period (d), distribution of path orientations for both control and for-knockdown flies before and after looming walking trials. Bar height indicate counts (e), fraction of flies fleeing for each looming presentation (f), proportion of flies performing each of the described behaviors (g) and (h). * denotes $p<0.05$. Sample sizes: control n = 64, *S6KII*-knocdown n = 63.

In **Figures 12g,h** we observe that the behavioral pattern displayed was very similar to the one of the previous conditions described: higher fraction of flies escaping in the beginning of the stimulation period, followed by an increase in the freezing fraction and a reduction in escaping fraction. As for the other two conditions (where *rut* and *for* were knockdown), the increase in freezing relative to the decrease in escapes, occurred much faster for the controls than for the *S6KII*-knockdown flies. Regarding the fraction of non-responder flies, we observed that this value is higher for the S6KII-knockdown flies although it becomes very similar to the controls in the second half of the stimulation.

Besides the results regarding the walking speed during the stimulation, all the other quantifications regarding this experiment that were not statistically significant are also shown in **Annex B**.

In summary, these results show that flies with lower neuronal levels of S6KII display lower levels of freezing under the conditions of our paradigm and, additionally, no statistically significant changes regarding walking speed parameters during the stimulation period were found. These observations suggest that this gene might be creating a learning deficit which is manifested particularly in the freezing behavior. For the reason, this might be a promising gene to further studies regarding our main question.

Summarizing these three experiments, by reducing the neuronal expression of three different learning and memory genes, we were able to observe differences in the defensive responses to looming of these flies. Overall, we found that reduced expression of any of the genes resulted in a reduction in freezing, while the effects on walking speed were more variable between the three, although it is important to note that the tendency to increase escape attempts make this findings stronger and more reliable by suggesting that the animals are trying to adjust to the situation with different defensive strategies. Given the observed reduction in freezing upon knockdown of learning genes, we next asked if the opposite effect on freezing was observed upon their overexpression, reasoning that in this condition learning should be facilitated. To answer this question, we studied defensive behaviors in flies in which we overexpressed *rut*. We chose this gene because this is the gene that has been more well described in learning and memory studies in *Drosophila*.

Effects of *rut* overexpression on *D. melanogaster* defensive behaviors

Given the observed reduction in freezing upon knockdown, we next asked if the opposite effect on freezing was observed upon overexpression, reasoning that in this condition learning should be facilitated. To answer this question, we studied defensive behaviors in flies in which we overexpressed *rut.* Specifically, we used the nysb-GAL4 driver line to drive the expression of an UAS-*rut* sequence across all the neuronal cells of the flies. As a control we used an empty-GAL4 driver line.

Figure 13 depicts the behavior of the *rut*-overexpression flies in a paradigm with a 5-minute-baseline. The fraction of flies freezing was higher throughout the stimulation period for the nsyb-GAL4/UAS-*rut* flies (see **Figure 13a**) and by the end we observed a significantly higher fraction of nsyb-GAL4/UAS-*rut* flies freezing (41/52) compared to controls (25/52) (z-test for independent proportions, $p < 0.01$). Moreover, the percentage of time spent freezing during stimulation was much higher for the nsyb-GAL4/UAS-*rut* flies (control: median = 9.0833 %, IQR = [0.5833; 65.375]; nsyb-GAL4/UAS-*rut*: median = 80.0%; IQR = [43.4999; 89.2500]. Kruskal-Wallis test, $p < 0.001$, see **Figure 13b**). This very low levels of freezing displayed by the control flies were not expected. We are not aware of any specific reason why this might have happened, although we can speculate that EmptyGAL4 might have a non-specific effect of some sort in these flies. Since this apparent effect might be the reason that we are indeed observing a higher levels of freezing for the flies overexpressing *rut*, we should find alternatives regarding the control of this experiment: maybe expression of exogenous gene, as we did in the previous knockdown experiments.

Figure 13c depicts the walking speed of both conditions throughout the experimental time. Curiously, nsyb-GAL4/UAS-*rut* flies displayed a substantially lower walking speed compared to controls both during baseline (control: median = 11.6066 mm/s, IQR = [10.1636; 12.5928]; nsyb-GAL4/UAS-*rut*: median = 8.2915 mm/s, IQR = [7.4036; 9.3453]. Kruskal-Wallis test, $p < 0.001$, see **Figure 13d**) and during stimulation (control: median = 15.736 mm/s, IQR = [13.7855; 17.0525]; nsyb-GAL4/UAS-*rut*: median = 10.9975 mm/s, IQR = [9.5874; 14.2729]. Kruskal-Wallis test, $p < 0.001$, see **Figure 13e**). The speed before the looming was also much lower for the flies overexpressing *rut* (control: median = 14.3259 mm/s, IQR = [12.8423; 16.7719]; nsyb-GAL4/UAS-*rut*: median = 10.3466 mm/s, IQR = [7.7630; 11.8240]. Kruskal-Wallis test, $p < 0.001$, see **Figure 13f**). As previously mentioned, the walking speed a fly has by the time of stimulus presentation can increase or decrease their probability of

freezing. These low values of walking speed displayed by the flies overexpressing *rut* might be the reason why they are showing higher levels of freezing during the stimulation period, since, in principle they would be more prompted to it. Additionally, we did not find any difference between the two conditions regarding the increase in speed in response to looming, suggesting that the escape vigor is not affected by *rut* overexpression, despite the other observed modulations in walking speed.

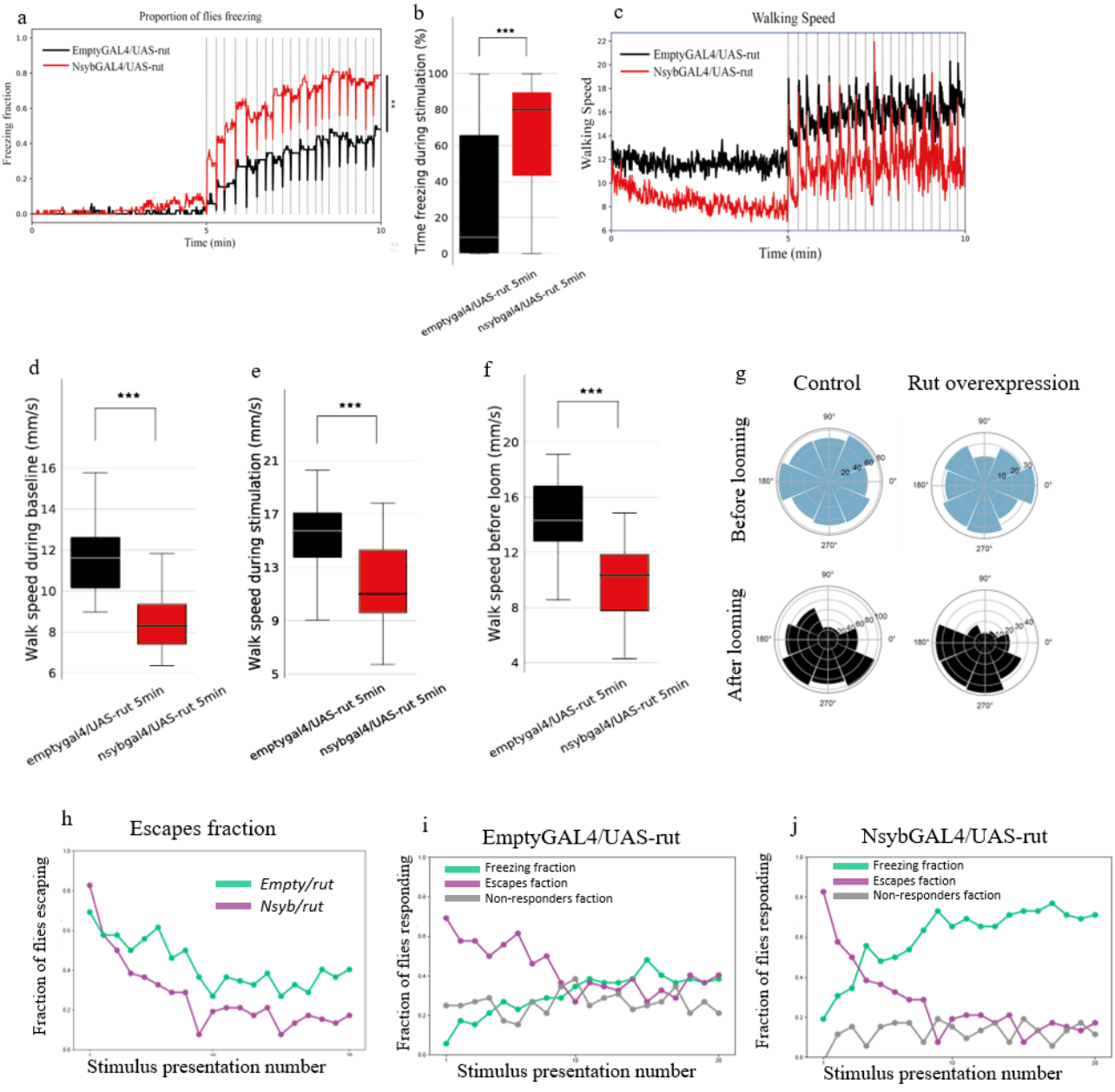

Figure 13. Behavioral analysis of flies overexpressing *rut*. Proportion of flies freezing. Dashed lines indicate stimulus presentations (a), percentage of time spent freezing during the stimulation period (b), average walking speed across the experiment (c), walking speed during baseline period (d), walking speed during stimulation period (e), walking speed before the looming presentation (f), distribution of path orientations for both control and *S6KII-*

knockdown flies before and after looming walking trials. Bar height indicate counts (g), fraction of flies fleeing for each looming presentation (h), proportion of flies performing each of the described behaviors (i) and (j). ** denotes p<0.01; *** denotes p<0.01. Sample sizes: control n = 52, *rut*-overexpression n = 52.

Moreover, we found significantly different walking orientations before and after the stimulus for both control flies and test flies (Mann-Whitney test, $p < 0.001$, see **Figure 13g**) indicating they were moving further away from the source of threat. **Figure 13h** shows that the nsyb-GAL4/UAS-*rut* flies displayed a lower escaping fraction than controls during the stimulation period, which is consistent with the higher fraction of flies freezing for that condition.

Finally, **Figures 13i,j** show that in both conditions there was a bigger fraction of flies escaping in the beginning of the stimulation and as the stimulation progressed, an increase in the freezing fraction and a reduction in the escaping fraction was observed. In this case, the increase in the freezing fraction relative to the decrease in escapes was faster for the flies overexpressing *rut*, as opposed to what was observed in the knockdown experiments. Contrary to what we observed in the previous experiments, the fraction of non-responder flies was lower for the flies overexpressing *rut* than for controls.

All the quantifications regarding this experiment that were not statistically significant are shown in **Annex C**.

In summary, these results show that flies overexpressing *rut*, display, indeed, higher levels of freezing throughout the stimulation. This findings support our hypothesis in the sense that show that flies with higher doses of a gene that is related to learning might have the processes related to this ability facilitated, this way, learning faster the inescapable properties of the arena and therefore, engage more in the more adaptive behavior: freezing. Nevertheless, we found that these animals had very low walking speed values which might be the reason why we are observing these higher levels of freezing. It is important to disambiguate this possibility.

D. melanogaster mutants' defensive behaviors

Although the use of RNAi offers some advantages, mainly the fact that we can target the knockdown specifically to neuronal cells, the use of mutants allows us to more carefully address how different doses of the relevant genes impact upon the selection of defensive responses. In the following experiments, we tested the effect of different doses of the *rut* and *for* genes and asked how these backgrounds with different doses responded to manipulations in baseline. For these experiments, we used the rut^{2080} allele (a P-element insertion upstream of the transcription start site) and three different conditions: homozygous mutants (rut^{2080}),

heterozygous mutants (rut^{2080}/+) created by crossing rut^{2080} with CS flies, and, wild-type CS flies as a control.

rut mutants

5-minute-baseline

Figure 14 depicts the behavior of the *rut* mutant flies in a paradigm with a 5-minute-baseline. Although we did not show any analysis regarding the freezing fraction or about the percentage of time spent freezing during baseline (see Methods section), it is important to note that, in this particular experiment, we observed that CS flies displayed very high levels of freezing during this initial period, however, we are not aware of the reasons why this happened, yet. As we can see in **Figure 14a**, during stimulation, the levels of freezing observed varied with the number of copies of *rut,* such that freezing was lowest for rut^{2080}, followed by rut^{2080}/+ and CS flies. By the end of the experiment, there were, indeed less rut^{2080} flies freezing (41/81) compared to the rut^{2080}/+ (58/75) and to the CS flies (75/83). This fraction was significantly lower for rut^{2080}/+ compared to CS flies (z-test for independent proportions, $p < 0.05$), and also significantly lower for the rut^{2080} compared to both CS (z-test for independent proportions, $p < 0.001$) and rut^{2080}/+ flies (z-test for independent proportions, $p<0.001$). Moreover, the percentage of time spent freezing during stimulation also showed a *rut* dose-dependent modulation, and was significantly different among conditions (CS: median = 89.5 %, IQR = [76.333; 94.0]; rut^{2080}/+: median = 81.1667%; IQR = [48.6667; 90.3338]; rut^{2080}: median = 15.5%, IQR = [0.3333; 71.8333]. Kruskal-Wallis test, $p < 0.001$, see **Figure 14b**), such that it was significantly lower for rut^{2080} compared to both CS (Dunn test, $p < 0.001$) and rut^{2080}/+ (Dunn test, $p < 0.001$), and also significantly lower for rut^{2080}/+ compared to CS (Dunn test, $p < 0.05$).

Figure 14c depicts the walking speed of all conditions throughout the experimental time. rut^{2080}/+ displayed higher values of walking speed during the baseline period (CS: median = 9.7192 mm/s, IQR = [8.7373; 10.3925]; rut^{2080}/+: median = 10.8373 mm/s, IQR = [9.4555; 11.8527]; rut^{2080}: median = 9.4628 mm/s, IQR = [8.4856; 10.2134]. Kruskal-Wallis test, $p < 0.001$, see **Figure 14d**), being significantly different from CS (Dunn test, $p < 0.001$) and rut^{2080} (Dunn test, $p < 0.001$). During the stimulation period, rut^{2080} flies displayed lower levels of walking speed (CS: median = 14.8072 mm/s, IQR = [12.4303; 16.9392]; rut^{2080}/+: median = 15.4469 mm/s, IQR = [12.7672; 17.7346]; rut^{2080}: median = 12.1475 mm/s, IQR = [10.9908; 12.9056]. Kruskal-Wallis test, $p < 0.001$, see **Figure 14e**), which were statistically different

from CS (Dunn test, $p < 0.001$) and from *rut*^{2080}/+ flies (Dunn test, $p < 0.001$). The difference in walking speed between stimulation and baseline reflected the increase in speed observed in response to looming for all conditions, (CS: median = 4.2426 mm/s, IQR = [2.2716; 7.1583]; *rut*^{2080}/+: median = 4.0625 mm/s, IQR = [1.9032; 7.0366]; *rut*^{2080}: median = 2.4989 mm/s, IQR = [1.8675; 3.2644]. Kruskal-Wallis test, $p < 0.001$, see **Figure 14f**), but that difference was significantly lower for *rut*^{2080} flies compared to CS controls (Dunn test, $p < 0.001$) and also compared to *rut*^{2080}/+ (Dunn test, $p < 0.001$). The speed before the looming was also significantly different among conditions (CS: median = 8.7009 mm/s, IQR = [6.1813; 11.3185]; *rut*^{2080}/+: median = 11.3593 mm/s, IQR = [8.4190; 13.0985]; *rut*^{2080}: median = 10.8848 mm/s, IQR = [9.3599; 12.0935]. Kruskal-Wallis test, $p < 0.01$, see **Figure 14g**) being significantly higher for *rut*^{2080}/+ and *rut*^{2080} when compared to CS flies (Dunn test, $p < 0.01$). All conditions under study increased their speed after looming, however, this increase was also significantly different among certain conditions (CS: median = 5.3592 mm/s , IQR = [3.4406; 8.742]; *rut*^{2080}/+: median = 4.0895 mm/s, IQR = [0.7919; 5.615]; *rut*^{2080}: median = 1.3236 mm/s, IQR = [-0.1995; 3.2747]. Kruskal-Wallis test, $p < 0.001$, see **Figure 14h**). This change in speed was significantly lower for *rut*^{2080} compared both to CS (Dunn test, $p < 0.001$) and *rut*^{2080}/+ flies (Dunn test, $p < 0.05$). Despite the differences in walking speed, all conditions displayed a significant change in the walking orientations towards the side furthest away from the screen after the looming presentations (CS: Mann-Whitney test, $p < 0.05$; *rut*^{2080}/+: Mann-Whitney test, $p < 0.001$; *rut*^{2080}: Mann-Whitney test, $p < 0.001$, see **Figure 14i**).

Figure 14j shows the fraction of flies escaping followed the opposite trend of that observed for the fraction of flies freezing, such that by the end t *rut*^{2080} flies displayed the higher escaping fraction, followed by the *rut*^{2080}/+ and finally the CS controls.

Finally, **Figures 14k,l,m** show that in the beginning of the stimulation period, escapes are the most common observed defensive behaviors. As the stimulation continued, we observed and increase in freezing and a decrease in escape attempts, in all conditions. At a certain time point, we observed an inversion in the fraction of occurrence of these behaviors, where the freezing fraction becomes higher in comparison to the escapes fraction. However, this inversion point happened later in the stimulation period for the *rut*^{2080} compared with the *rut*^{2080}/+ and the and the CS flies. The fraction of non-responder flies was very low and similar for both CS and *rut*^{2080}/+ flies, however, *rut*^{2080} ones displayed higher values regarding this variable which become very similar to the fraction of escapes in the second half of the stimulation.

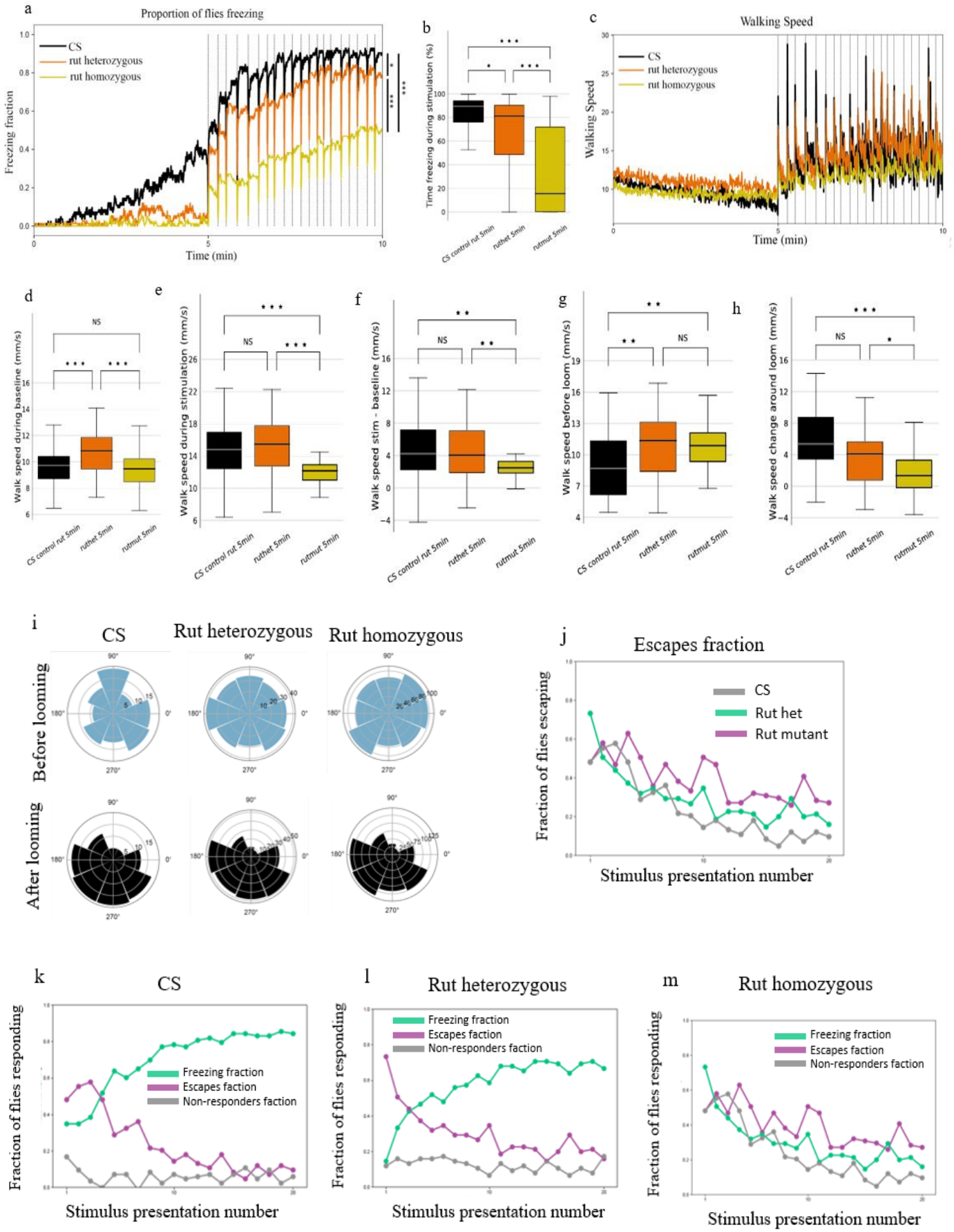
a
Proportion of flies freezing
CS
rut heterozygous
rut homozygous
Freezing fraction
Time (min)
b
Time freezing during stimulation (%)
CS control rut 5min
ruthet 5min
rutmut 5min
c
Walking Speed
CS
rut heterozygous
rut homozygous
Walking Speed
Time (min)
d
Walk speed during baseline (mm/s)
e
Walk speed during stimulation (mm/s)
f
Walk speed stim - baseline (mm/s)
g
Walk speed before loom (mm/s)
h
Walk speed change around loom (mm/s)
i
CS
Rut heterozygous
Rut homozygous
Before looming
After looming
j
Escapes fraction
CS
Rut het
Rut mutant
Fraction of flies escaping
Stimulus presentation number
k
CS
l
Rut heterozygous
m
Rut homozygous
Freezing fraction
Escapes faction
Non-responders faction
Fraction of flies responding
Stimulus presentation number

Figure 14. Behavioral analysis of *rut* mutants under a 5-minute-baseline paradigm. Proportion of flies freezing. Dashed lines indicate stimulus presentations (a), percentage of time spent freezing during the stimulation period (b), average walking speed across the experiment (c), walking speed during baseline period (d), walking speed during stimulation period (e), change in walking caused by looming presentation (baseline period subtracted from stimulation period) (f), walking speed before the looming presentation (g), change in walking speed caused by stimulus presentation (pre-stimulus period subtracted from post-stimulus period) (h), distribution of path orientations for both control and for-knockdown flies before and after looming walking trials. Bar height indicate counts (i), fraction of flies fleeing for each looming presentation (j), proportion of flies performing each of the described behaviors (k), (l) and (m). * denotes $p<0.05$; ** denotes $p<0.01$; *** denotes $p<0.01$; NS = not significant. Sample sizes: CS n = 83, *rut*/+ = 75, *rut* n = 84.

30-seconds-baseline

Next we reasoned that freezing behavior in flies with learning and memory defects should be more sensitive to manipulation in baseline duration, therefore, we expected to see that these flies were not going to be able to compensate their genetic deficit as much as they would probably do when a longer exploration time is provided, as in the previous experiment. Therefore, for flies with the same manipulations as before, we expected to see that the final levels of freezing upon the end of the experiment would be much lower for flies subjected to short baseline periods not only compared to controls. To test this hypothesis, we tested the genetic backgrounds with different *rut* doses in a paradigm in which we only allowed 30 seconds of baseline exploration, and looked at freezing levels.

Figure 15 depicts the behavior of the *rut* mutant flies in a paradigm with 30 seconds of baseline. In the beginning of the stimulation period, there were no substantial differences regarding the freezing behavior of all the three conditions. As the stimulation progressed a higher fraction of flies freezing was observed for CS flies in comparison to the homozygous and heterozygous condition. Further along the stimulation period, a difference could also be observed between heterozygous and homozygous, with the former displaying a higher freezing fraction than the latter (see **Figure 15a**). By the end of the experiment, we observed that there were indeed less rut^{2080} flies freezing (20/84) compared to rut^{2080}/+ (32/79) and to the CS flies (64/89). We next saw that this fraction was significantly lower for the rut^{2080}/+ compared to the CS (z-test for independent proportions, $p < 0.001$), significantly lower for the rut^{2080} compared to the CS (z-test for independent proportions, $p < 0.001$) and also significantly lower for rut^{2080} compared to rut^{2080}/+ (z-test for independent proportions, $p < 0.05$). Moreover, the percentage of time spent freezing during stimulation was lower for the rut^{2080} (CS: median = 38.6667 %, IQR = [6.3333; 58.6667]; rut^{2080}/+: median = 4.5% ; IQR = [0.0; 41.6667]; rut^{2080}: median = 0.5833%, IQR = [0.0; 9.2083]. Kruskal-Wallis test, $p < 0.001$, see **Figure 15b**). This variable

was significantly lower for *rut*^{2080}/+ compared to CS (Dunn test, $p < 0.001$) and significantly lower for *rut*^{2080} flies compared to CS (Dunn test, $p < 0.001$) and to *rut*^{2080}/+ (Dunn test, $p < 0.05$).

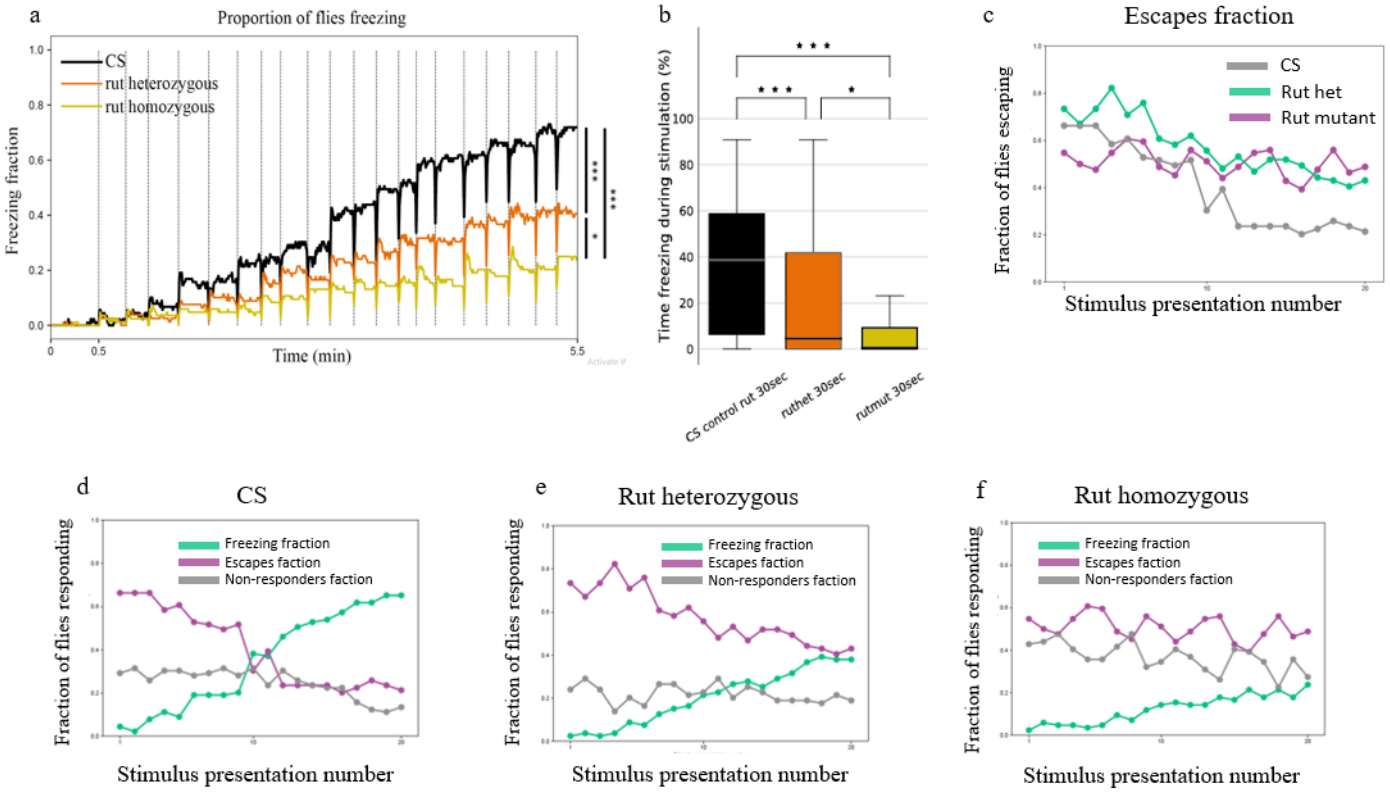

Figure 15. Behavioral analysis of *rut* mutants under a 30-second-baseline paradigm. Proportion of flies freezing. Dashed lines indicate stimulus presentations (a), percentage of time spent freezing during the stimulation period (b), fraction of flies fleeing for each looming presentation (c), proportion of flies performing each of the described behaviors (d), (e) and (f). * denotes $p<0.05$; *** denotes $p<0.01$. Sample sizes: CS n = 89, *rut*/+ n = 79, *rut* n = 81.

Figure 15c shows the fraction of flies escaping followed the opposite trend of that observed for the fraction of flies freezing, such that by the end *rut*^{2080} flies displayed the higher escaping fraction, followed by the *rut*^{2080}/+ and finally the CS controls, exactly as we observed in the previous experiment. Although, it is important to note that overall, the fraction of escapes for all conditions was higher than the values observed in the 5-minute-baseline paradigm. This supports the hypothesis that exploration time and therefore, the learning processes that might be happening during that time have an impact in the defensive behaviors displayed.

Interestingly, while the difference in the fraction of flies freezing between wild-type and heterozygous flies is rather small for the paradigm with 5 minutes of baseline, this difference becomes larger when the baseline is shortened, which is in agreement with our hypothesis that flies with learning and memory defects should be more sensitive to baseline manipulations. This effect can also be observed when we examine the plots that show the different behaviors together (see **Figures 15d,e,f**): in the paradigm with 5 minutes of baseline, after a certain number of looming stimuli, the fraction of flies freezing becomes higher than the fraction for flies escaping for both controls and heterozygous flies, however, in the paradigm with 30 seconds of baseline, such inversion is only observed for control flies, while for the heterozygous ones, the fraction of flies escaping remains higher throughout the entire stimulation period. The fraction of non-responder flies was very similar for both CS and rut^{2080}/+ flies although not as low as the one observed in the previous experience where rut^{2080} ones displayed higher values regarding this variable compared to the other two conditions.

All the quantifications regarding this experiment that were not statistically significant or not regarding to freezing behavior are shown in **Annex D**.

In summary, the results of these two experiments with *rut* mutants in which we manipulated the duration of the baseline period show that flies with different doses of *rut* gene display different levels of freezing among each other although both lower than control flies. The flies with a higher dose of this gene, the heterozygous ones, display higher levels of freezing compared to the homozygous mutants. The heterozygous mutants, when given more time to learn the context displayed levels of freezing very closed to the controls and, on the contrary, the homozygous, even when allowed to explore the arena for longer periods of time seemed to still be too compromised in terms of learning to be able to increase their levels of freezing. We also observed that in the experiment where we shortened the baseline duration flies with lower doses of the genes, both homozygous and heterozygous mutants, were more compromised by this shorter period of exploration than the controls.

Curiously, we found that unlike what was described above for controls and heterozygous mutants, which have very similar final levels of freezing in the longer baseline paradigm, the difference in freezing between heterozygous and homozygous mutants is larger in this experiment compared to the one where the flies only have 30 seconds of exploration. We believe the reason we observed this opposite effect relates to the very low levels of protein

expression in the homozygous mutant, which disparately affect freezing modulation by baseline manipulations in relation to the heterozygous flies. Thus, giving them more time to explore does not seem to compensate their learning deficits. Although not the main focus of our hypothesis, we also analyzed aspects related to locomotor behavior in the 30 seconds paradigm, and we found they were in large part similar to what we had found for the 5 mins baseline paradigm. As mentioned above, we show those results in **Annex D.**

for mutants

We focused on the for^{S} flies and not on the for^{R} ones, mentioned in the introduction of the present work because of practical reasons. We tried to run these experiments with for^{R} flies but they were extremely unhealthy and therefore very difficult to maintain. Moreover, the fact that they displayed this very unhealthy aspects also reinforced our choice of not using them for these experiments. Here, we studied the differences in defensive responses between homozygous (for^{S}) and heterozygous flies (for^{S}/+) under two different baseline conditions (30 seconds and 5 minutes).

for^{S} vs for^{S}/+ 30-second-baseline

Figure 16 depicts the behavior of the for^{S} and for^{S}/+ flies in a paradigm with a 30-second-baseline. In the beginning of the stimulation period, there were no apparent differences between the freezing fraction of the two conditions. In the second half of the stimulation period for^{S}/+ showed a higher freezing fraction than for^{S} flies (see **Figure 16a**), and by the end of the experiment we observed that there were significantly more for^{S}/+ flies freezing (23/80) compared to for^{S} (8/77) (z-test for independent proportions, $p < 0.01$).

Figure 16b shows the average walking speed of both conditions under study throughout the experimental time. for^{S} displayed significantly lower walking speed than for^{S}/+ flies both during baseline (for^{S}/+: median = 13.27 mm/s, IQR = [12.202; 14.3336]; for^{S}: median = 11.375 mm/s, IQR = [10.3496; 12.196]. Kruskal-Wallis test, $p < 0.001$, see **Figure 16c**) and stimulation (for^{S}/+: median = 14.5166 mm/s, IQR = [13.03; 15.868]; for^{S}: median = 12.697 mm/s, IQR = [11.747; 13.51]. Kruskal-Wallis test, $p < 0.001$, see **Figure 16d**). The speed before the looming was significantly lower for for^{S} flies (for^{S}/+: median = 12.44 mm/s, IQR = [11.4783; 13.749]; for^{S}: median = 10.93 mm/s, IQR = [10.09; 11.969]. Kruskal-wallis test, $p < 0.001$, see **Figure 16e**), which, once again would suggest that these flies were more prompt to freeze. However,

we observed the opposite, these flies barely freeze, which supports our hypothesis that this tendency might indeed me related to their learning deficits. Although both conditions increased their speed after the looming presentations, the change of speed around the looming was also significantly lower for *for*^{S} flies (*for*^{S}/+: median = 3.739 mm/s, IQR = [2.438; 5.2219]; *for*^{S}: median = 2.5157 mm/s, IQR = [1.44; 3.72]. Kruskal-Wallis test, $p < 0.001$, see **Figure 16f**). Despite these differences, the increase in walking speed during stimulation relative to the baseline was the same for both conditions. Furthermore, walking flies of both conditions changed their orientation away from the screen after the looming presentations (*for*^{S}/+: Mann-Whitney test, $p < 0.001$; *for*^{S}/+: Mann-Whitney test, $p < 0.001$).

Figure 16h shows that the escape fraction of both conditions was very high at the beginning of stimulation period. However, contrary to what has been observed in previous experiments, this values only slightly decreased during stimulation. Moreover, the escape fractions were very similar for both conditions throughout the whole period.

Lastly, **Figures 16i,j** show that in the beginning of stimulation period, the great majority of the flies were displaying escape responses and the freezing fraction was very reduced for both conditions. As opposed to what we have found in other experiments, as the stimulation continued, we did not observe a sharp decrease in the escape fraction with accompanying sharp increase in the freezing fraction We could observe that a trend for this pattern was present in the plot referring to *for*^{S}/+ flies, although in a really subtle way. On the contrary, *for*^{S} flies, apart from a slight initial reduction in the escapes fraction, showed values that seemed constant across the stimulation period, and no tendency was observed for an increase in freezing and a decrease in escapes. For both genotypes the escapes fraction remained higher than the freezing fraction throughout the stimulation period. The fraction of non-responder flies was slightly higher than the fraction of flies freezing and very similar for both *for*^{S}/+ and *for*^{S}. Although, it is important to remembered that we did not used wild-type flies as a control in these experiments, which might have facilitated the interpretation of these results. The addition of this control is in our future plans.

All the quantifications regarding this experiment that were not statistically significant are shown in **Annex E.**

In summary, these experiments showed that both *for*^{S}/+ and *for*^{S} flies display low levels of freezing while subject to our stimulation paradigm not significantly different from each other. Moreover, these flies have very high escapes fractions which suggests that they

are still engaging in a defensive strategy although is not the more adequate regarding the context.

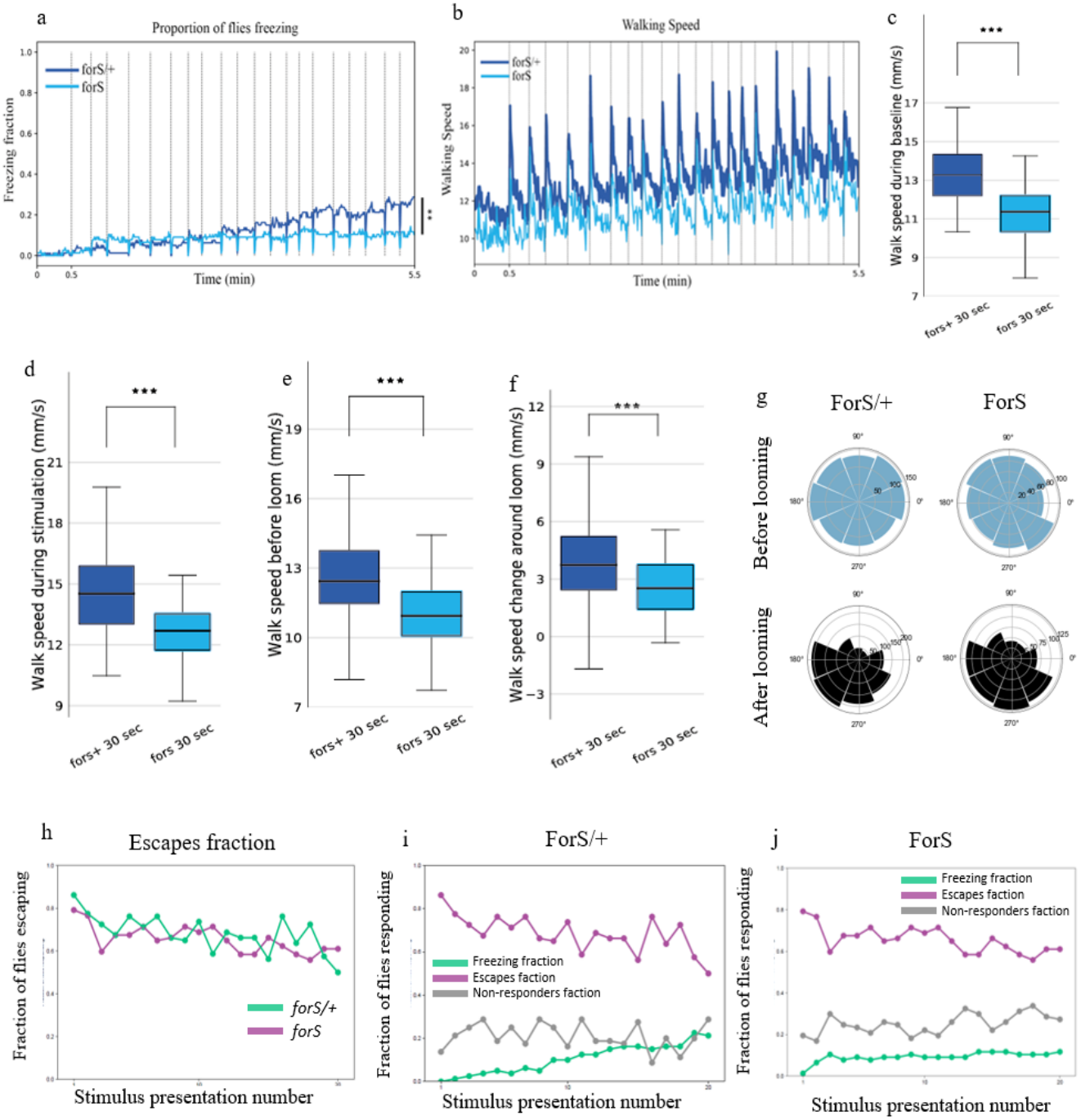

Figure 16. Behavioral analysis of for^{S} flies under a 30-second-baseline paradigm. Proportion of flies freezing. Dashed lines indicate stimulus presentations (a), average walking speed across the experiment (b), walking speed during baseline period (c), walking speed during stimulation period (d), walking speed before the looming presentation (e), change in walking speed caused by stimulus presentation (pre-stimulus period subtracted from post-stimulus period) (f), distribution of path orientations for both control and for-knockdown flies before and after looming walking trials. Bar height indicate counts (g), fraction of flies fleeing for each looming presentation (h), proportion of flies performing each of the described behaviors (i) and (j). ** *denotes p<0.01;* *** *denotes p<0.01. Sample sizes*: for^{S}/+ n = 80, for^{S} n = 77.

for^S vs for^S/+ 5-minute-baseline

The fact that by the end of the stimulation period, the freezing fraction displayed by *for*^{S}/+ flies seemed to be showing a tendency to increase (see **Figure 16a**), led us to hypothesize that if more time was given to these flies to explore the arena, a significant difference in freezing between *for*^{S} and *for*^{S}/+ flies would be observed. Thus, we decided to run the exact same experiment, this time using a longer baseline period and we looked at the differences in the freezing behavior.

Figure 17 depicts the behavior of the *for*^{S} and *for*^{S}/+ flies in a paradigm with a 5-minute-baseline. When the stimulation started, very pronounced differences regarding the freezing behavior of these flies arose. *for*^{S} flies showed lower freezing fractions during the entire stimulation period (see **Figure 17a**) and by the end of the experiment, there were, indeed, significantly less *for*^{S} flies freezing (2/78) compared to *for*^{S}/+ (39/80) (z-test for independent proportions, $p < 0.001$). Moreover, the percentage of time spent freezing during stimulation was also lower for *for*^{S} flies (*for*^{S}/+: median = 28.83%, IQR = [0.1667; 72.958]; *for*^{S}: median = 0.0%; IQR = [0.0; 0.6667]. Kruskal-Wallis test, $p < 0.001$, see **Figure 17b**).

Regarding the walking speed very similar patterns were observed compared to the previous experiment for the variables under study (**Annex F**). **Figure 17c** shows that, both conditions were displaying higher and very similar escaping fractions, in the beginning of the stimulation period, but as the stimulation continued, this fraction sharply decreased for *for*^{S}/+ flies as opposed to what we observed in the previous experiment with the shorter baseline. On the contrary, *for*^{S} flies a decrease in their escape fraction keeping it at high relatively constant values.

Finally, **Figures 17d,e** shows that at the beginning of the stimulation period, the majority of the flies were displaying escape responses and the freezing fraction was very reduced on both conditions. As the stimulation continued, we observed an increase in freezing and a decrease in escape attempts for *for*^{S}/+ flies, as opposed to *for*^{S} flies where the escapes fraction remained higher than the freezing fraction throughout the stimulation period. In the second half of the stimulation period the fraction of escapes for *for*^{S}/+ flies became lower than their freezing fraction contrary to what we observed in the previous experiment with a shorter baseline duration, which supports our hypothesis that by giving more time for the flies to explore the context freezing tends to be the preferred behavioral strategy. The fraction of non-responder flies was slightly higher for *for*^{S} flies than for *for*^{S}/+ ones although in the first case,

it is important to note that, the non-responders fraction is higher than the fraction of flies freezing.

All the quantifications regarding this experiment that were not statistically significant, as well as the results regarding the walking speed, are shown in **Annex F.**

In summary, by increasing the baseline duration in these experiments we observed a totally different behavior regarding the freezing levels of *for*^{S}/+ flies. Again, this suggests that different gene-doses allied to different exploration times can modulate in a great extent the behavioral defensive patterns of animals with learning impairments, as we had previously showed in the *rut* mutants' experiments.

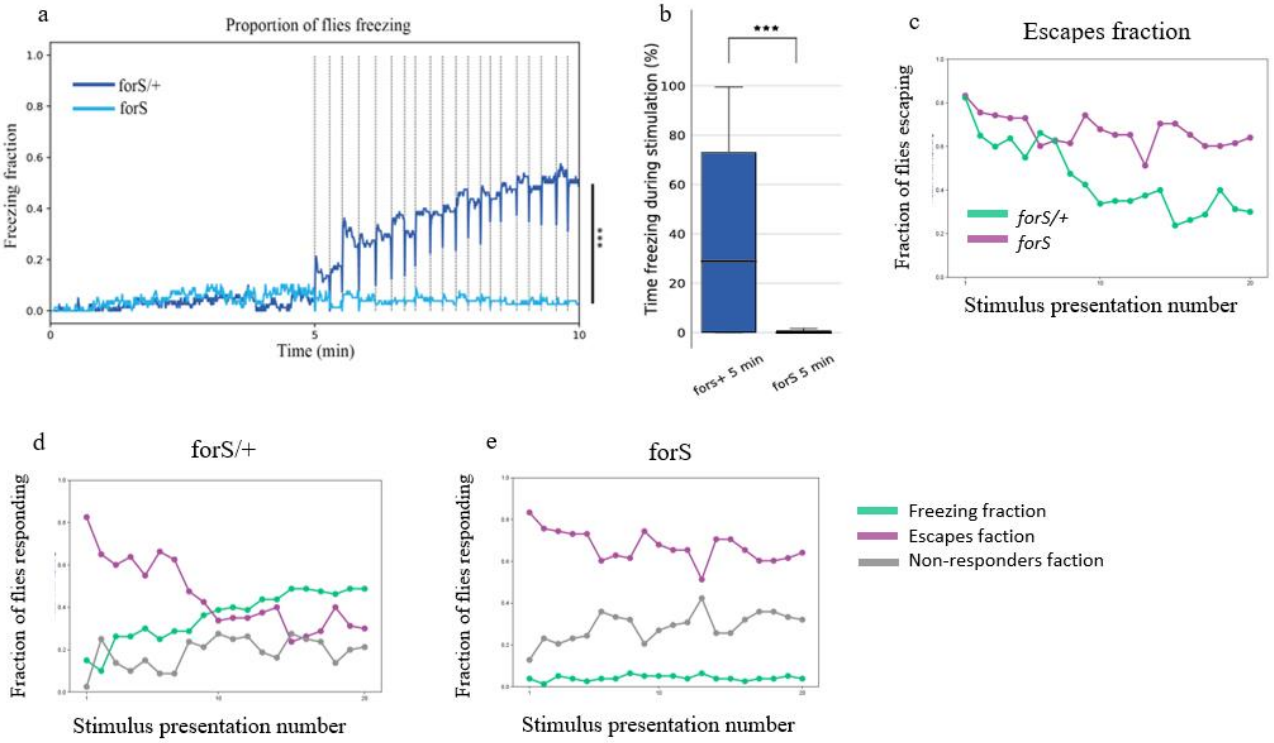

Figure 17. Behavioral analysis of for^{S} flies under a 5-minute-baseline paradigm. Proportion of flies freezing. Dashed lines indicate stimulus presentations (a), percentage of time spent freezing during the stimulation period (b), fraction of flies fleeing for each looming presentation (c), proportion of flies performing each of the described behaviors (d) and (e). *** denotes p<0.01. Sample sizes: for^{S}/+ n = 80, for^{S} n = 78.

By running these experiments with mutants for our genes of interest, we were able to recapitulate the results obtained with the RNAi experiments. In both cases, manipulated flies displayed lower levels of freezing compared to controls which, once again, supports our initial hypothesis that learning about the environment is important for the defensive behavioral choice and can indeed modulate it. Therefore, the recapitulation that the mutant experiments did of our RNAi experiments make our results more reliable and supported.

Discussion

Defensive behaviors are crucial for survival. Predation is constantly present in a prey ecological time and it is probably one of the strongest evolutionary forces regarding species perpetuation, however, individuals have the ability to perceive, evaluate and take behavioral decisions about this risk and in order to overcome it (Lima & Dill, 1990): Freeze, flight or fight. Therefore, selecting the most adaptive defensive strategy upon a threatening situation is very important. Nevertheless, a major question still remains: How does an animal select which defensive behavior to engage in? Very little is known about how this process of selection occurs and how the individual's context can influence it. Therefore, we were interested in study how the knowledge about the spatial context could modulate the selection of defensive behaviors in *D. melanogaster*.

The animal's surroundings have been shown to be an important variable when it comes to decide what to do upon a threatening situation, especially the presence or absence of a refuge (de Oca et al., 2007; Vale et al., 2017). Moreover, it has also been reported that when animals are allowed to explore an inescapable environment before encountering a threat, freezing becomes their preferred behavioral choice (Robert J. Blanchard et al., 1976). This exploration period seems to help the animal to get familiar with its inescapable context and therefore to choose the most adequate behavioral response.

Similarly, in the case of fruit flies, in order to know whether or not there is, for instance, a shelter nearby, they need to learn about their environment. Therefore, supported by the findings regarding mice mentioned above, a fly should flee until it learns that there is no escape and once the inescapable properties of the spatial context are perceived, the preferred and most adaptive behavior should be freezing. Hence, we hypothesized that freezing would be the behavioral strategy most likely to be affected by differential levels of context familiarity when escape is not possible. Consequently, the present work aimed to bring to light new knowledge about how defensive behaviors of *D. melanogaster* can be modulated by context familiarity about the experimental arena, and in particularly, get a better understanding on how learning about the animal's surroundings modulates their freezing behavior.

In order to study this topic, we manipulated the expression of genes involved in memory and learning processes. We expected the animals that suffered these manipulations to have impairments on those abilities. Thus, the learning process that was supposed to happen during baseline (L. Liu et al., 2007; Zacarias et al., 2018) should not happen in the same extent in these

flies with learning and memory defects, as it would occur in normal healthy ones. This compromised ability to learn the inescapable properties of the arena should result in different choices regarding defensive behaviors in comparison with flies in which both memory and learning abilities were not compromised in any manner. Contrary, by overexpressing *rut*, we expected to facilitate the cognitive abilities regarding memory and learning in which this gene is required. Thus, in this case, we expected flies to learn about the surroundings in a more efficient and faster way than controls and therefore, adopt the more adaptive defensive strategies according to the context, in this case, freezing.

We found that flies in which *rut*, *for* and *S6KII* genes were not properly expressed, displayed significantly different behaviors compared to controls, mostly regarding freezing. We have shown that flies with reduced expression of the previously mentioned genes, displayed reduced levels of freezing compared to controls. On the contrary, we showed that the overexpression of *rut* led to higher levels of freezing compared to controls. Besides these findings regarding freezing behavior, we have also reported that the manipulations mentioned above caused changes in walking speed, especially in response to stimulation. Furthermore, we found that these impaired flies that froze less were displayed a lot of escape attempts suggesting a compensation mechanism regarding their defensive strategies. Although our analysis regarding escapes is still qualitative and very much preliminary should not be disregarded in the interpretation of our results.

Freezing

It has been proposed that some form of learning takes place during the baseline period of these experiments (Zacarias, 2019) and, as mentioned above, the choice between fleeing and freezing, in rodents, has been shown to be strongly dependent of the spatial context (de Oca et al., 2007; Vale et al., 2017). When we knockdown *rut*, *for* and *S6KII* genes in *D. melanogaster*, we observed reduced levels of freezing during the stimulation period, for all of them, compared to controls. These results are in concordance with our initial hypothesis that by not properly expressing genes related to learning and memory, the behavioral output observed under our paradigm would be different with a higher tendency to affect the level of freezing of these flies. Moreover, these results support the idea that there is some form of learning happening during the baseline (Zacarias et al., 2018). Disrupting these flies' ability to learn the inescapability properties of the arena results in a switch in the selection of defensive strategies, such that flies

adopt the least adaptive strategy given the inescapable properties of their surrounding environment.

The cues that fruit flies use during this baseline period in order to get familiar with it are not totally understood yet. In the wild, these animals use the sun to help them navigate through the environment (Giraldo et al., 2018), they use path integration mechanisms during food search to keep close to the food source (El Jundi, 2017) and have specific neurons, that encode for their head direction, based on internal and external sensory cues, and for spatial contextual cues, such as, rotation and direction (Varga & Ritzmann, 2016). However, in our experimental assays, these variables are difficult to assess. Regarding our limitations and scenario, an obvious sensory modality that flies can be using to perceive the space is vision, however they can also be using additional sources of information during this exploratory phase. We observed, as Zacarias and colleagues (2018) also noticed, that flies, during baseline, walked mostly along the round walls of the arenas. This tendency to remain close to the walls and edges is associated with a shelter-seeking behavior (Laurent Salazar, Planas-Sitjà, Sempo, & Deneubourg, 2018) suggesting that flies are exploring their environment during baseline. This tendency has been shown to decrease in familiar environments and to increase under stressful situations (Durier & Rivault, 2003) which ultimately suggests that besides vision, tactile information might also play an important role during exploration.

The experiment where we overexpressed *rut* was idealized from the hypothesis hat these flies, by having a higher dose of *rut* gene would have their learning and memory processes facilitated. Indeed, we observed that these flies displayed higher levels of freezing than controls which, once again, supports our hypothesis that, the better the learning performance during baseline, the higher the probability of knowing the absence of an escape or of a shelter, engaging, therefore, in freezing. However, we still have to be aware of the fact that the animals overexpressing *rut* showed significantly lower walking speeds, which can justify the higher levels of freezing detected (Zacarias et al., 2018). This subject will be approached in more detail ahead on the present work.

Regarding the experiments with the rut^{2080} mutants, where we used two different baseline durations (5 minutes and 30 seconds), we observed that the more copies of the gene these animals had, the higher the levels of freezing they displayed, supporting the hypothesis that this learning gene's function modulates the behavioral choices do *D. melanogaster*. We asked how genetic backgrounds with different *rut* gene doses responded to manipulations in baseline duration. Our hypothesis was that learning and memory genetic sensitized

backgrounds should display a stronger modulation of defensive responses upon changes in exploration time. Therefore, we expected greater differences in comparison to controls regarding freezing behavior between flies in the 30-second-baseline protocol compared to the 5-minute-baseline one.

We focused on *rut*^{2080}/+ whose freezing in the 5-minute baseline paradigm was only slightly lower compared to *wild-type* controls. Interestingly, the differences regarding this variable between these two types of flies were higher when they were subjected to a shorter baseline period. We plan to statistically analyze the effect size of this differences, in the future, to quantitatively support this statement. These results suggest that, the lack of one gene's copy seems to be more compromising when these flies had less exploration time compared to the case in which they had a longer exploration period, and therefore, more time to learn about the context. Once again, these results reinforce the existence of learning processes occurring during the baseline. In the longer baseline scenario, *rut*^{2080}/+ and the control flies displayed, indeed, very similar freezing fractions supporting the idea that the lack of one gene's copy does not constitute such a relevant compromise when enough time to explore the context was provided to the animals. We then looked at the same differences but this time between *rut*^{2080}/+ and *rut*^{2080}. In this case we saw that when giving *rut*^{2080} a longer exploration period, the difference between these mutants and *rut*^{2080}/+ regarding freezing fraction was even higher than the one observed in the 30-second-baseline paradigm. These *rut*^{2080} mutants probably have a much more severe impairment since that even when giving them more time to explore the environment they are compromised in such a great extent that it is not enough to compensate their deficits.

Lastly, the fact that *for*^{S}/+ flies displayed totally different freezing levels depending on the baseline period duration supports in a very strong way, the hypothesis that there are indeed learning processes occurring during this initial period of exploration and that the information acquired during the baseline seems to modulate the defensive behaviors displayed during the stimulation period. It is important to note that we still want to standardize the experiments regarding these mutants by adding a CS control as we did for the *rut*^{2080} mutant ones.

Importantly, the mutant's results on this topic recapitulate the findings obtained with the RNAi lines.

Probability of freezing

According to the Zacarias and colleagues (2018) findings regarding the speed-dependent regulation of defensive behaviors in *D. melanogaster*, there is a relationship between freezing probability and the speed at which the flies are moving before looming presentation, such that, the higher the speed, the lower the probability they will freeze in response to that looming.

As reported in the results section of the present work, several of our manipulations resulted in lower walking speed before looming: *rut*-knockdown, rut^{2080} (for the 30-second-baseline paradigm) and *for* mutants (for both baselines). According to Zacarias and colleagues (2018) findings mentioned above, these lower speeds displayed by these flies would increase the probability of them reacting to looming by freezing leading, this way, to higher levels of freezing displayed by these flies overall. However, we observed exactly the opposite: all of these flies displayed lower levels of freezing compared to controls. Moreover, we saw that the probability of freezing at lower speeds was lower for these flies than for the controls (**Annex G**). Thus, being these flies, in theory more prone to freeze, the fact that we observed the exact opposite, gives strength to the hypothesis that these animals are indeed freezing less because of a learning impairment and the modulations that it induces on their defensive behavior's selection. Even though, these results seem to support a learning impairment, further experiments would be needed in order to disentangle this hypothesis. One option to demonstrate that this reduced freezing actually results from a learning impairment would be to silence the relevant neurons regarding this process during baseline and observe how the flies would behavior during stimulation with their activity unaltered. However, we are still not aware of which neurons are important and involved in this process. Nevertheless, this can still be an interesting route to pursue.

In the case of the *rut* overexpression experiments, we found substantial differences in walking speeds, with lower walking speed for flies with significantly lower walking speeds for the flies overexpressing *rut* in all neurons., Contrary to the previous mentioned experiments, we observed that the probability of freezing for lower speeds was much higher for these flies than for the controls (**Annex G**). These results raised the possibility that these flies were only freezing more because of this low velocities that the displayed (Zacarias et al., 2018). Therefore, is of major importance to take this analysis to a higher level in order to undertint what is going on with these animals. Thus, to tease apart the contribution of the low walking speeds for the

high levels of freezing observed, we would need to test the same flies in a closed-loop paradigm, in which looming presentations would be contingent on the walking speed of the flies. This would allow us to more directly measure freezing probabilities across different walking speeds. Additionally, we could also try to run an experiment where we express *dunce*-RNAi in a pan-neuronal manner. *dunce* is a gene that has also been implicated in memory and learning processes in fruit flies which encodes a cAMP phosphodiesterase (Davis, Cherry, Dauwalder, Han, & Skoulakis, 1995; Qiu et al., 1991) and whose under expression, therefore, might biochemically resemble the overexpression of *rut*, in the sense that the degradation of cAMP would be defective and as in an overexpression of *rutabaga* scenario, this molecule would have higher intracellular levels than normal conditions. Another option to better understand this situation would be to run experiments where we manipulate the Rac1 and Raf genes. Rac1 pathway is involved in the active forgetting of labile memory and Raf pathway provides and active protection of this formed memory (X. Zhang, Li, Wang, Liu, & Zhong, 2018). Therefore, by either knockdown the expression of rac1 gene or by overexpressing Raf we could be able to create conditions, biochemically similar to the overexpression of *rut* and try to disambiguate the results we got. Nevertheless, there were also detected opposite situations where flies displayed higher speeds before looming presentations compared to controls (*for*-knockdown, rut^{2080} and rut^{2080}/+ mutants (5-minute-baseline), and rut^{2080}/+ mutants (30-seconds-baseline). In all these cases, the freezing levels displayed by these flies were lower compared to controls which could raise the option that these animals were just freezing less because of their higher walking speeds before the loomings (Zacarias et al., 2018). Apart from the rut^{2080}/+ mutants in the 30-second-baseline protocol, which displayed very similar freezing probabilities at higher speeds compared to controls (**Annex G**), the remaining cases mentioned above displayed lower freezing probabilities at higher speeds compared to controls (**Annex G**). These flies might indeed be displaying lower levels of freezing because of the higher walking speeds they have before the looming presentations (Zacarias et al., 2018) which ultimately would not support our hypothesis that their lower levels of freezing are caused by their learning deficits. To further understand what is going on in these sets of flies we could also run this same experiment in the closed-loop paradigm previously mentioned, to measure the probability of these flies freezing when displaying higher walking speeds and evaluate if on those cases they show lower probabilities of freezing than the control flies.

Are the flies perceiving the stimulus?

One possible explanation to the reduction in freezing we observed, could be related to a defect in stimulus perception. However, we observed that flies used in our experiments besides increasing their speed after being presented with looming, which is an indication of a vigorous escape attempt, changed their orientation further away from the threat source after the stimulation delivery. Both these results suggest that, these flies were indeed perceiving and reacting to the stimuli. This information strengthens up the hypothesis that the altered defensive choices of these individuals are due to something other than not being able to perceive the stimulus. Moreover, this data suggests that, the non-freezing flies were probably displaying escape attempts away from the stimulus, upon stimulation.

Escapes

As mentioned above, flies in an inescapable environment, should flee until they learn that there is no escape. Once the inescapable properties of the spatial context are perceived, the preferred and most adaptive behavior should be freezing. If it is true that flies only freeze after learning that there is no escape, when we compromise their learning abilities, the reduction in freezing should be accompanied by an increase in escapes. Hence, we hypothesized that flies that freeze less will tend to display more escape attempts. Indeed, as mentioned before, we were aware that flies that were not freezing were still reacting to the stimulus. Further analysis allowed us to understand that these non-freezing flies were, in fact, displaying other forms of defensive behaviors instead.

The fraction of flies freezing was lower for all the knockdown flies and for both rut^{2080} and for^{S} mutants, regardless the baseline duration protocol, compared to controls during stimulation. However, we observed that all of these flies displayed higher escapes fractions during that same period relatively to controls which suggests that, although they had a lower tendency to respond to looming with freezing, they were still adjusting their response by performing other forms of defensive behaviors, in this case, escape attempts. The mutant results on this topic recapitulate the findings obtained with the RNAi lines.

Regarding the experiments where we overexpressed *rut*, the results were exactly the opposite. Since these flies displayed higher levels of freezing, supposedly because they were learning about their context faster and better, there were few flies escaping, mostly because the majority of them were freezing. Thus, the results mentioned before about RNAi knockdowns

and mutants seemed to be coherent with the results of the overexpression experiments where we observed the opposite as expected.

Both results supported our hypothesis that the reduced levels of freezing should be accompanied by an increase in escape attempts.

Freezing, Escaping, Non-responding

Freezing and escaping are mutually exclusive defensive strategies (Eilam, 2005). Thus, when engaging in one of these behaviors, an animal cannot perform the other one simultaneously. If freezing tends to be chosen when there is no escape, then the default should be to flee until one learn that escape is not possible (Zacarias et al., 2018). Therefore, we expected that if the fraction of flies freezing increases, the fraction of flies escaping will most certainly decrease, unless flies are not responding to the stimulus anymore.

When we plotted the freezing fraction together with the escapes fraction, we observed that a decrease in the former was indeed accompanied by an increase in the latter. In the beginning of the stimulation, we always found the escapes fraction to be higher than the freezing fraction. In the 5-minute-baseline paradigm, we observed that the fraction of flies escaping gradually increased with each looming, and at a certain point during the stimulation period became higher than the fraction of flies escaping. Importantly, for the flies with reduced dose of learning and memory genes, this increase in the freezing fraction relative to escapes fraction, was always slower than for the respective controls. As a result, the point at which the number of flies freezing becomes larger than the number of flies escaping – which I will refer to as inversion point – occurs later during the stimulation for the knockdown and mutant flies, suggesting a slower learning process. Additionally, this effect was dose-dependent, such that for *rut* homozygous mutants, the inversion point occurs later than for heterozygous mutants. In fact, *rut* heterozygous flies display a behavior that is close to the one observed for the wild-type controls, which suggests that having only one copy of the gene can be sufficient, provided we give the flies a long period of time to explore the arena. However, when subjected these flies to a shorter baseline period, the fraction of flies escaping remained higher than the fraction of flies freezing throughout the entire stimulation period for both homozygous and heterozygous *rut* flies, while this was not the case for controls. Again, these observations suggest that genetic backgrounds with reduced copies of learning and memory genes, are more sensitive to the reduction of baseline duration i.e. the time allowed for exploration of the surrounding context prior to stimulus presentation. Besides these observations, it is important to note that, rut^{2080}

homozygous flies showed a relatively high fraction of flies non-responding which can suggest that these flies might have other impairments that prevent them from reacting to the stimulus than we initially thought. Further experiments and analysis should be done to understand better this issue. On option would be to do a conditional rescue of this gene only in the brain. If we still saw a high fraction of non-responder flies that would suggest that this inability to respond to the stimulus is not due to a learning deficit but rather to some other sort of impairment. If, on the other hand, we managed to decrease the amount of non-responder flies, that would suggest that their inability to react to the stimuli was probably due to some learning deficit. In this latter case, we could keep doing conditional rescues in other parts of the animal's body to try to understand from where the lack of response might be coming from. In either case, another approach we could try after the rescues would be to isolate the data from the non-responder flies and analyze just for the behavioral parameters that we are evaluating and try to understand which patterns are observed and what is going on, especially regarding walking speed and locomotor parameters not only to figure out if they are showing some type of altered behavior upon stimulation but also to be sure that they are, indeed, perceiving the stimulus.

The behavior of for^{S} flies was similar to the one of rut^{2080} mutants. When under a 30-second-minute baseline protocol, both for^{S} and $for^{S}/+$ displayed rather high escape levels accompanied by virtually no freezing behavior. Their elevated levels of escapes were not surprising since both these flies displayed low freezing fractions during this period, which perfectly justifies the fact that they might be compensating this by performing other types of defensive behaviors instead. These high values of escapes and low values of freezing were maintained throughout the whole stimulation, and no inversion between these behaviors' fractions happened. The absence of that pattern might have to do not only with the learning impairment these flies have, but also with the short baseline paradigm, which they were being subjected to which did not allow them to explore the context for enough time. When under a 5-minute-baseline paradigm, we observed that $for^{S}/+$ flies displayed a different pattern regarding the freezing and escaping fractions and the inversion point between them indeed happened. Here, we observed that for the same type of flies, by increasing the duration of the baseline period, this inversion point was actually observed although it only happened halfway through the stimulation period. The differences detected, regarding this last analysis, between the present experiment and the previous one with a shorter baseline period, were probably due to the behavioral compromise that arose when subjecting these flies to a short baseline scenario. In the case of for^{S} mutants, we did not observe a tendency for a decreasing escape fraction and

an increase freezing fraction, as expected, since these flies displayed very low levels of freezing during the entire stimulation. Thus, no inversion point was observed and ultimately, the pattern observed did not suggest that this inversion would eventually happen if we gave more time for the flies to explore the arena prior to stimulation. On the other hand, these findings support the idea that although displaying very low levels of freezing during the stimulation period, for^{S} flies seemed to maintain their escape attempts at high levels during that time, suggesting an adaptive choice of alternative defense behaviors.

Finally, regarding the *rut* overexpression experiments we observed that, flies inverted the escape and freezing fractions earlier on stimulation compared to controls. Keeping in mind the previous suggested hypothesis that whoever inverts these behavioral fractions first is learning faster, these results suggest that these manipulated flies were indeed learning faster about their spatial context than controls and therefore, adopting the most adaptive behavior earlier on.

With the mutants and overexpressing experiments we had further evidence that indeed baseline period, and gene dosage, are important factors for the flies to be able to get to know their environment and that depending on that period and that gene dosage, different patterns of defensive behaviors can be observed. Furthermore, these observations gave support to the fact that the context familiarity might be modulating the defensive behavioral choice of these animals. Moreover, the fact that we observed totally opposite results between the RNAi experiments, in which we reduced the expression of *rut*, and the experiments in which we overexpressed this gene, give strength to the importance of this gene and of its dosage regarding the modulation of the behavioral choices involved in the paradigm. To further support this results we should try to replicate these findings with the other two genes we have been working with.

Future directions

Habituation to the arena as a *proxy* for learning

Habituation is a form of learning in which an organism decreases its response to repeated stimuli (Harris, 1943). As Zacarias and colleagues (2018) described wild-type flies tend to decrease their average walking speed throughout the baseline period, and this decrease is thought to reflect habituation to the environment during baseline exploration. Therefore, we

hypothesized that flies with learning and memory deficits would be impaired in this habituation process, and hence, show a lower reduction in walking speed during baseline.

We observed that flies with reduced levels of *rut* seemed to display this decrease in walking speed during the baseline period. Thus, the fact that these flies seem to be habituating to the arena might raise doubts regarding whether there is indeed a learning deficit regarding what we observed afterwards, during the stimulation. However, since the manipulations regarding this gene seem to have pronounced effects on the walking speed of these flies, we cannot be sure that these observations are entirely related to an eventual habituation process to the arena. Moreover, this fact, makes the it harder to interpret the sharp decrease of walking speed observed during the baseline of the *rut*-overexpression experiment, even if this decrease may suggest a faster habituation to the arena.

On the contrary, for^{S} homozygous flies did not decrease their walking speed during baseline regardless the baseline duration protocol used. This might suggest that the habituation process to a novel environment is compromised in these flies. However, for the $for^{S}/+$ flies this decreased was observed in both baseline durations. These observations support the hypothesis that different gene-dosage is leading to a different behavioral pattern, and in this case, suggesting different levels of learning deficits.

In the experiments not mentioned above, a decrease in walking speed during baseline did not seem to be detected. However, since this are observations done by visual inspection of the plots, further proper quantitative analysis would be needed not only in the cases where did not seem to be a decrease in walking speed during baseline but also in the cases where this tendency seemed to exist. We found interesting to pursue this line of though since habituation if also a form of learning and therefore by understanding better in what extent these impaired flies can habituate to the arena, we might be able to better understand these flies' ongoing learning processes. To further analyze this topic, we can either do (1) a linear regression of the walking speeds of these flies during the initial part of the baseline period, e.g the first 30 seconds, since after that the decrease in walking speed is very smooth (Soibam et al., 2012) and using the data from the whole baseline period would probably dilute the effect we are searching for; (2) piece-wise linear regression so we could use the whole baseline period and maybe even investigate the differences between the initial phase of habituation and the more later smoother one; or (3) use an exponential regression as another option to be able to use the entire baseline but having only one set of parameters (Soibam et al., 2012).

Preliminary experiments regarding the Central Complex

The Central Complex (CX), as mentioned before, in the introduction, has been implicated in learning and memory and more specifically in spatial memory (Pfeiffer & Homberg, 2014; Stern et al., 2019). Several different studies addressed the contribution of specific regions of the CX for spatial learning and memory. For instance, The F1 and F5 neurons of the FB, were found to mediate specific visual pattern memory features in *D. melanogaster* (G. Liu et al., 2006) and the R2 and R4m neurons of the EB, were discovered to be also important for visual memory but independently of visual patterns (Pan et al., 2009; Stern et al., 2019).

With the goal of identifying particular structures and/or specific neurons from CX relevant for the acquisition of the spatial environment familiarity in the context of defensive behavior selection, we have run preliminary experiments where we manipulated neuronal activity or gene expression in specific regions of the CS.

Specifically, we silenced the F5 neurons of the FB (c205-GAL4) resorting to the GAL4/UAS system, only in adult flies, and assessed how such silencing affected the selection of defensive responses. Although Liu and colleagues (2006) found a role for these neurons in visual pattern memory, no phenotype regarding the freezing behavior was observed in our experiments (**Annex H**). This might suggest that F5 neurons are important for visual pattern memory but not for the spatial memory that we believe is required under our experimental paradigm. Another option is that these flies might, indeed, use visual pattern memory during our assay but can also be using other processes more important for this task to learn about the context. Other neurons, that we still did not identify, might be more prone to affect the performance of these flies under these circumstances. Furthermore, Stern and colleagues (2019) showed that besides CX, MBs also need to be functional during visual learning spatial task in fruit flies. Although we have not studied the contributions of MBs for our assay, it is possible that this structure also plays a role in this learning process.

Simultaneously to the previous experiment where we silenced the F5 neurons, we decided to also explore, again in a preliminary way, the effects of reduced expression of *rut*, *for* and *S6KII* specifically in these neurons (**Annex H**). For that we expressed RNAi of these genes using the same GAL4 line (c205-GAL4). We saw that reduced expression of *rut* in these neurons did not show a phenotype regarding to reduced freezing compared to controls, which contrasts to the finding of Liu and colleagues (2006) regarding the need of this gene in F5 neurons for a non-impaired visual pattern memory. Regarding the reduced expression of *for*

and *S6KII* on F5 neurons no conclusions could be drawn regarding freezing behavior, since we believe our controls were not working as supposed. Finally, we also explored the effects of a reduced expression of *for* and *S6KII* in the R3 and R4m neurons of the EB. For that we used a GAL4 line that labels these neurons (c819-GAL4) and the same procedure previously mentioned (**Annex H**). Unfortunately, the levels of freezing observed for the controls used were very low, which prevented us from taking any conclusions regarding the role of these neurons in defensive behavior selection. We would like to try this experiment also with *rut* since it has also been reported to play an important role in visual pattern memory (Pan et al., 2009) and therefore, might be a good candidate gene to investigate under our paradigm, because might be involved in other types of visual memory required for our paradigm linked to these EB neurons, contrary to what we observed for *for* and *S*6KII.

Thus, in the future we would like to continue to explore the CX regions related to learning and memory that might be involved in the modulation of defensive behaviors in the fruit fly resorting to the silencing tools we have been using (**Annex H**). We have already tried to silence several different regions of the CX, using different GAL4 lines, in order to explore their functions regarding the learning and memory processes that might be modulating Drosophila's defensive behaviors. However, most of the lines we silenced did not have progeny which is indicates that the silencing of these neurons during development is lethal and therefore, no viable adults were available to test under our paradigm. An experimental alternative to solve this issue is to use $GAL80^{ts}$, which allows us to silence the neurons of interest only in adulthood, avoiding, this way, that the silencing becomes lethal. We have already started the experiments using this tool, the silencing of the F5 neurons of the FB mentioned above was done using this protocol, although this process is much slower and laborious. Another alternative that we still want to persue is to try to silence these CX neurons resorting to optogenetics since the temporal resolution of this technique is much higher and thereby the obtained results can be more accurate. Silencing these neurons with optogenetic techniques instead of using permanent methods allows us to do the silencing only during the baseline period and therefore disentangle more easily what are the contributions of these neurons to the learning processes that we believe are happening during this initial period. Importantly, using optogenetic silencing allows us to leave neuronal activity intact during the exposure to the looming stimuli, such that any effect on freezing should not result from deficits in looming detection or motor expression of freezing.

Finally, we would also like to continue to try to outline which genes are important in specific CX regions. Since the use of RNAi knockdowns regarding these last experiments

seemed to work poorly, we might end up trying out other approaches, for instance, use mutants for our genes of interest and use the GAL4/UAS system to rescue those genes in the CX regions that we are studying. This way, instead of quantifying the behavior of flies with lower levels of the gene, compared to flies that have normal levels of it, we quantify the behavior of flies that have the gene only on those neurons, compared to flies that do not have that gene.

Conclusion

Most of the studies regarding freezing as a defensive behavior were done in mammals until very recently. Nonetheless, the prevalence of this behavior across such distant taxa suggests an analogous evolutionary process which ultimately strongly supports its adaptive value. Given this, studying this in a more tractable organism for which a huge number of tools are available to dissect neural circuits underlying behavior, studies regarding defensive behaviors in invertebrates started arising, especially using the fruit fly as a model organism. However, most of the first studies allowed flies to escape upon stimulation, and because of that, freezing was not a behavior commonly observed. As the studies using inescapable environments started to be done, freezing could be observed and studied. Still, a big question remained: How does an animal choose between freezing or fleeing when facing a threat?

In the present work we found that fruit flies with learning and memory defects froze less than controls when exposed to inescapable looming, while still displaying escapes although less robust. When we manipulated genes related to memory and learning, we observed dose-dependent effects in freezing: heterozygous mutants displayed an intermediate phenotype between WT flies and homozygous mutants. These dose-dependent effects were also observed upon baseline's duration manipulation.

The flies we worked with had their ability to learn and memorize about their surroundings compromised, and therefore the level of context familiarity they could attain was expected to be lower than controls. Our results support our initial hypothesis that lower levels of context familiarity should result in reduced freezing. Moreover, the fact that we manipulated three different genes related to memory and learning and we obtained reduced levels of freezing behavior in all of them, supports even more the hypothesis that this altered behavior is being caused by the flies' impairments regarding these cognitive abilities.

Understanding the mechanisms that support these action's selection and modulation involves a big compromise between internal and external factors that is difficult to tackle and

unravel but that will definitely play a role on these decision-making processes with huge ethological value. The present work aimed to bring to light new knowledge about the complex neural underpinnings that underly these decision processes and that ultimately ensure that animals engage in the most adaptive behavior according to the situation avoiding being predated or attacked because “Being killed greatly reduce future fitness” (Lima & Dill, 1990), and at the end of the day, ground rules of biology say that are the fittest ones that stay here to tell the story, and most important, to procreate.

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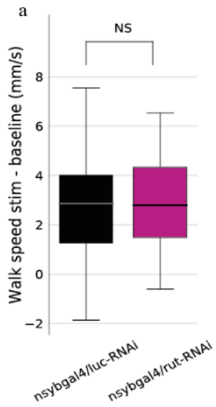
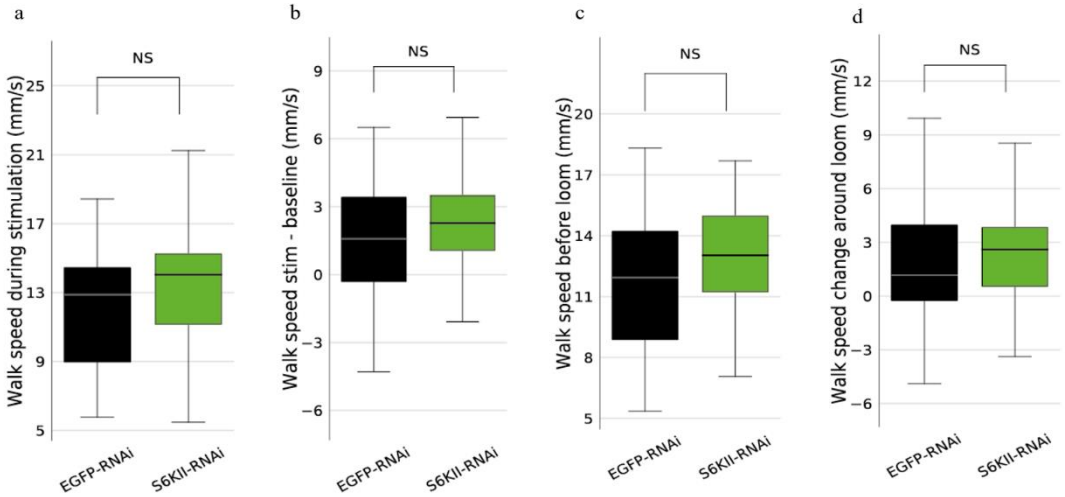
Annexes

A. *rut*-knockdown experiment.



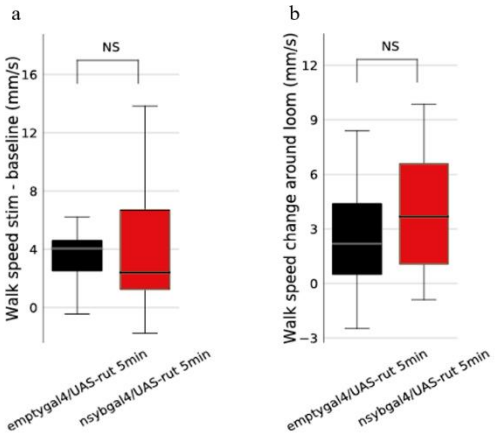
A1. *rut*-knockdown non-statistically significant results. Walking speed during stimulation period (control: median = 2.8601 mm/s, IQR = [1.2589; 4.0129]; *rut*-RNAi: median = 2.7884 mm/s, IQR = [1.4882; 4.43359]. Kruskal Wallis test, p = 0.5581) (a). NS = not significant.

B. *S6KII*-knockdown experiment.



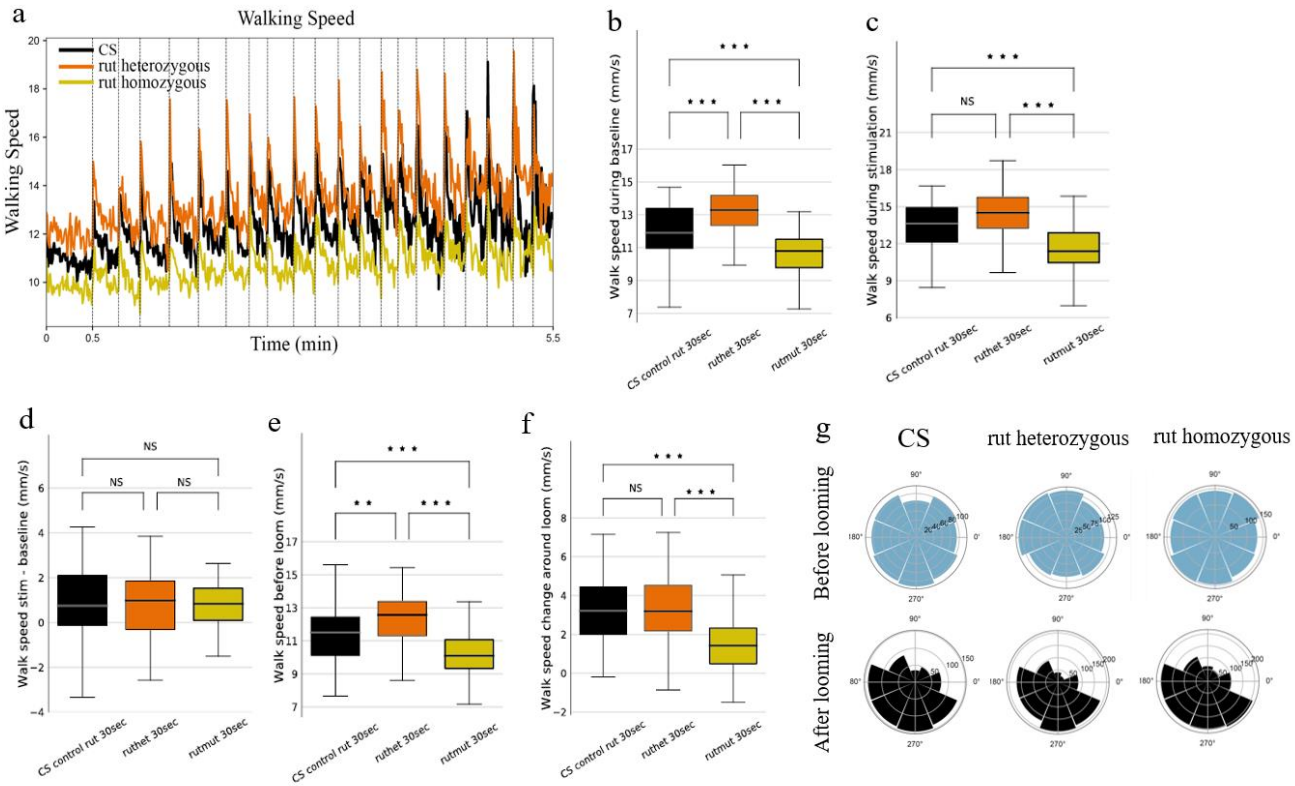
A2. *S6KII*-knockdown non-statistically significant results. Walking speed during stimulation period (control: median = 12.87 mm/s, IQR = [8.99; 14.411]; *S6KII*-RNAi: median = 14.04 mm/s, IQR = [11.167; 15.225]. Kruskal-Wallis test, p = 0.103) (a), change in walking speed caused by the looming presentation (baseline period subtracted from stimulation period) (control: median = 1.5869 mm/s, IQR = [-0.292; 3.3997]; *S6KII*-RNAi flies: median = 2.274 mm/s, IQR = [1.0765; 3.485]. Kruskal Wallis test, p = 0.337) (b), walking speed before the looming presentation (control: median = 11.933 mm/s , IQR = [8.898; 14.1955]; *S6KII*-RNAi: median = 13.038 mm/s, IQR = [11.2496; 14.948]. Kruskal-wallis test, p = 0.120) (c), average walking speed change around the looming time point (control: median = 1.17 mm/s, IQR = [-0.224; 3.943]; *S6KII*-RNAi: median = 2.599 mm/s, IQR = [0.5627; 3.819]. Kruskal-wallis test, p = 0.3478) (d). NS = not significant.

C. *rut* overexpression experiment.



A3. *rut* overexpression non-statistically significant results. Change in walking speed caused by the looming presentation (baseline period subtracted from stimulation period) (control: median = 4.0605 mm/s, IQR = [2.5568; 4.5708]; nsyb-GAL4/UAS-*rut*: median = 2.4039 mm/s, IQR = [1.2724; 6.6849]. Kruskal Wallis test, p = 0.1435) (a), change in walking speed caused by stimulus presentation (pre-stimulus period subtracted from post-stimulus period) (control: median = 2.1913 mm/s, IQR = 0.5154; 4.3688]; nsyb-GAL4/UAS-*rut*: median = 3.6716 mm/s, IQR = [1.0824; 6.5772]. Kruskal-wallis test, p = 0.0713) (b). NS = not significant.

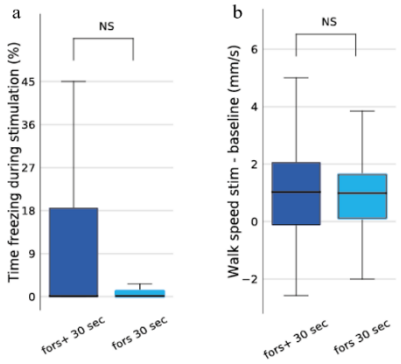
D. *rut* mutants (30 seconds baseline) experiment.



A4. Non-statistically significant results regarding the *rut* mutants experiment (30 second baseline) and the walking speed parameters not mentioned in the main text. Walking speed during across the experiment. Dashed lines

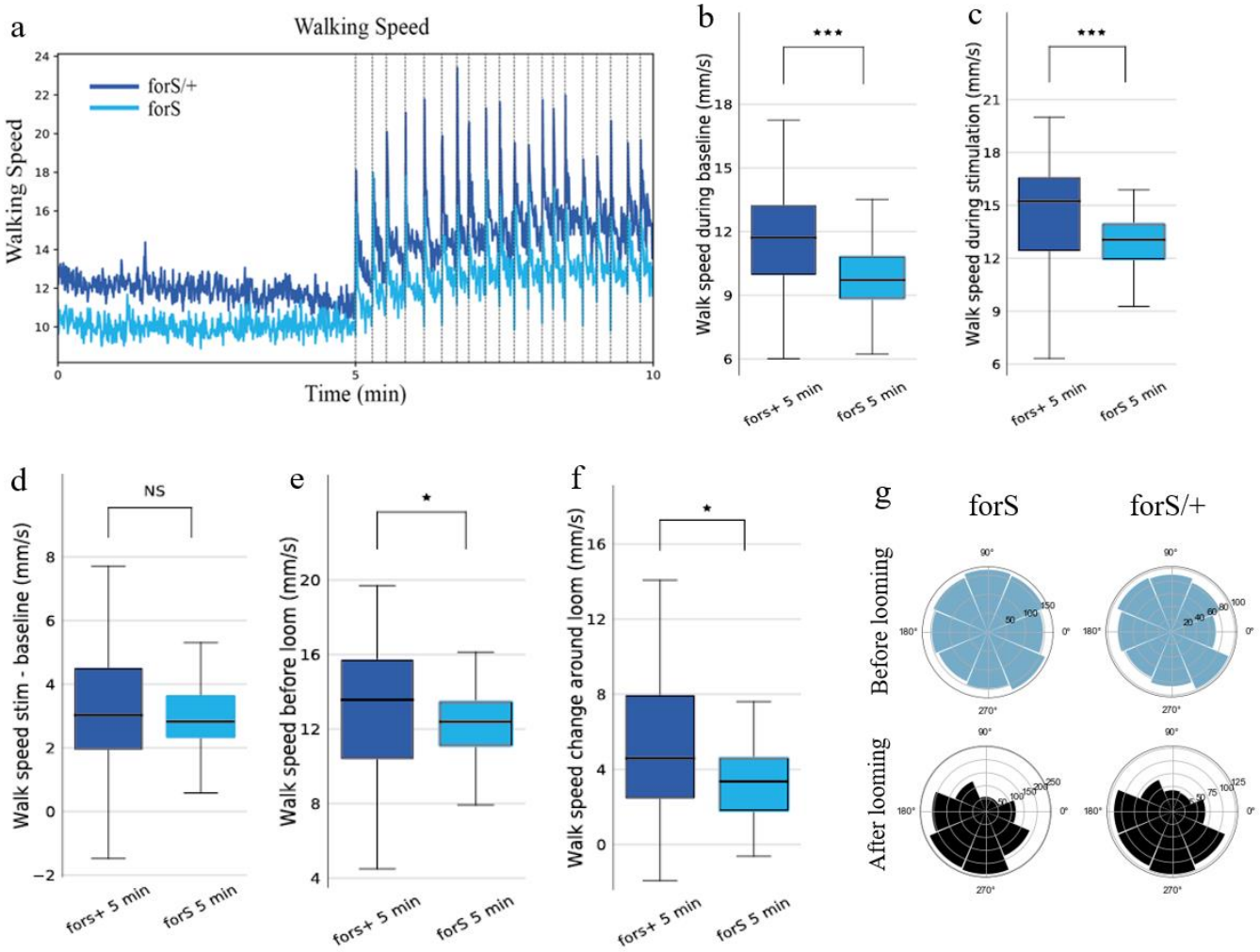
indicate stimulus presentations (a), walking speed during baseline (CS: median = 11.8947 mm/s, IQR = [10.9634; 13.383]; *rut*^{2080}/+: median = 13.2869 mm/s, IQR = [12.3593; 14;1608]; *rut*^{2080}: median = 10.7929, IQR = [9.7802; 11.5050]. Kruskal-Wallis test, $p < 0.001$) (b), walking speed during stimulation (CS: median = 13.6334 mm/s, IQR = [12.1331; 1409156]; *rut*^{2080}/+: median = 14.4934 mm/s, IQR = [13.2711; 15.7334]; *rut*^{2080}: median = 11.3889 mm/s, IQR = [10.4607; 12.8702]. Kruskal-Wallis test, $p < 0.001$) (c), change in walking speed caused by the looming presentation (baseline period subtracted from stimulation period) (CS: median = 13.6334 mm/s, IQR = [12.1331; 1409156]; *rut*^{2080}/+: median = 14.4934 mm/s, IQR = [13.2711; 15.7334]; *rut*^{2080}: median = 11.3889 mm/s, IQR = [10.4607; 12.8702]. Kruskal-Wallis test, $p < 0.001$ (d), walking speed before the looming presentation (CS: median = 11.5099 mm/s , IQR = [10.1265; 12.4318]; *rut*^{2080}/+: median = 12.5698 mm/s, IQR = [11.3146; 13.3657]; *rut*^{2080}: median = 10.0989 mm/s, IQR = [9.3363; 11.0630]. Kruskal-Wallis test, $p < 0.001$) (e), change in walking speed caused by stimulus presentation (pre-stimulus period subtracted from post-stimulus period) (CS: median = 3.2191 mm/s , IQR = [1.9918; 4.4443]; rut/+: median = 3.1963 mm/s, IQR = [2.1854; 4.5146]; *rut*: median = 1.49271 mm/s, IQR = [0.4927; 2.3224]. Kruskal-Wallis test, $p < 0.001$) (f), distribution of path orientations for both control and *for*-knockdown flies before and after looming walking trials. Bar height indicate counts (CS: Mann-Whitney test, $p < 0.001$; *rut*^{2080}/+: Mann-Whitney test, $p < 0.001$; *rut*^{2080}: Mann-Whitney test, $p < 0.001$) (g). ** denotes $p<0.01$; *** denotes $p<0.01$; NS = not significant.

E. *for*^{S} mutants (30 seconds baseline) experiment.



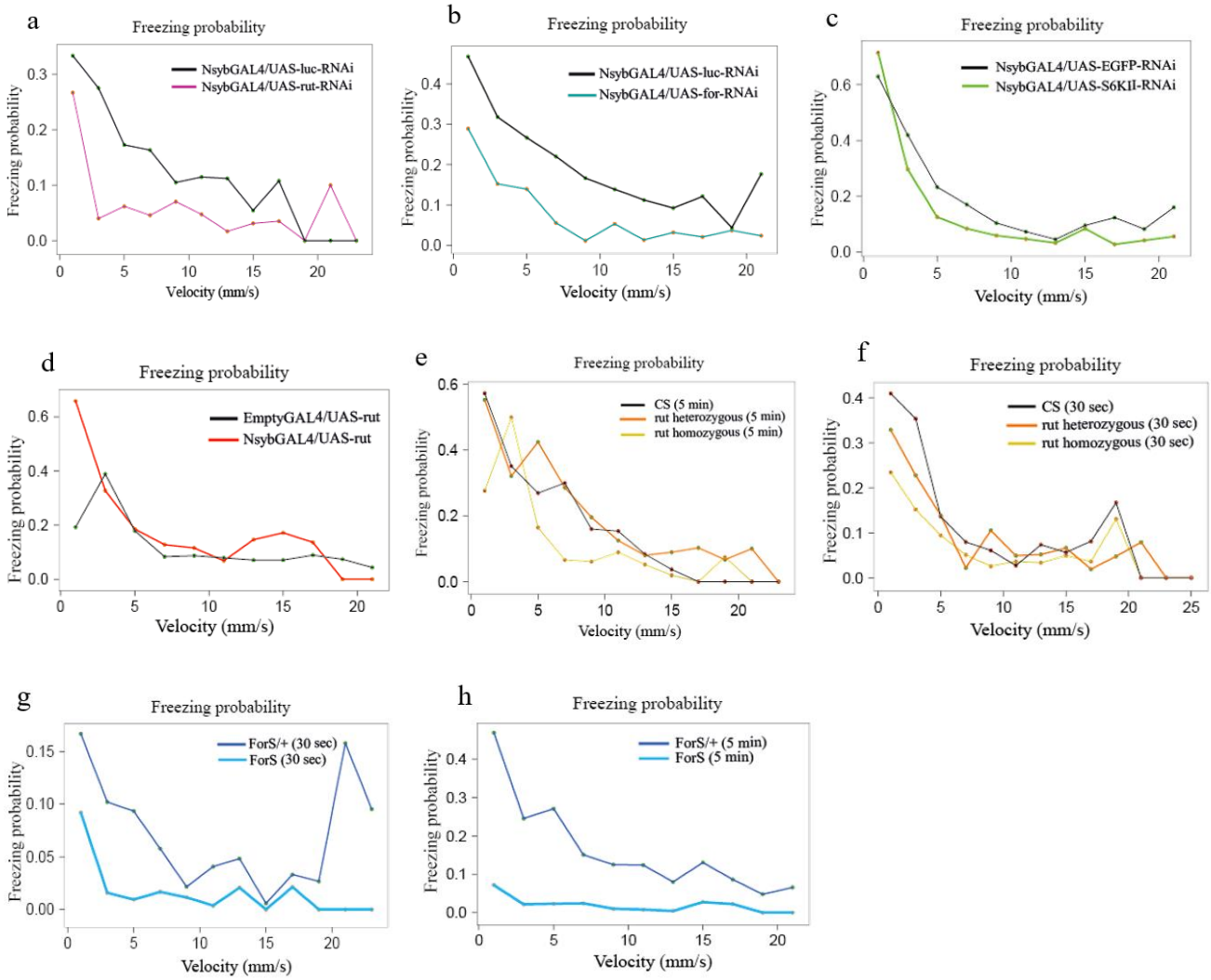
A5. Non-statistically significant results regarding the *for*S flies experiment (30 seconds baseline). Percentage of time spent freezing during stimulation period (*for*^{S}/+: median = 0.166%, IQR = [0.0; 18.458]; *for*^{S}: median = 0.1667%; IQR = [0.0; 1.1667]. Kruskal-Wallis test, $p = 0.4928$) (a), change in walking speed caused by the looming presentation (baseline period subtracted from stimulation period) (*for*^{S}/+: median = 1.02 mm/s, IQR = [-0.10; 2.04]; *for*^{S}: median = 0.992 mm/s, IQR = [0.12; 1.63]. Kruskal Wallis test, $p = 1.0$) (b). NS = not significant.

F. *for*^{S} mutants (5 minutes baseline) experiment.



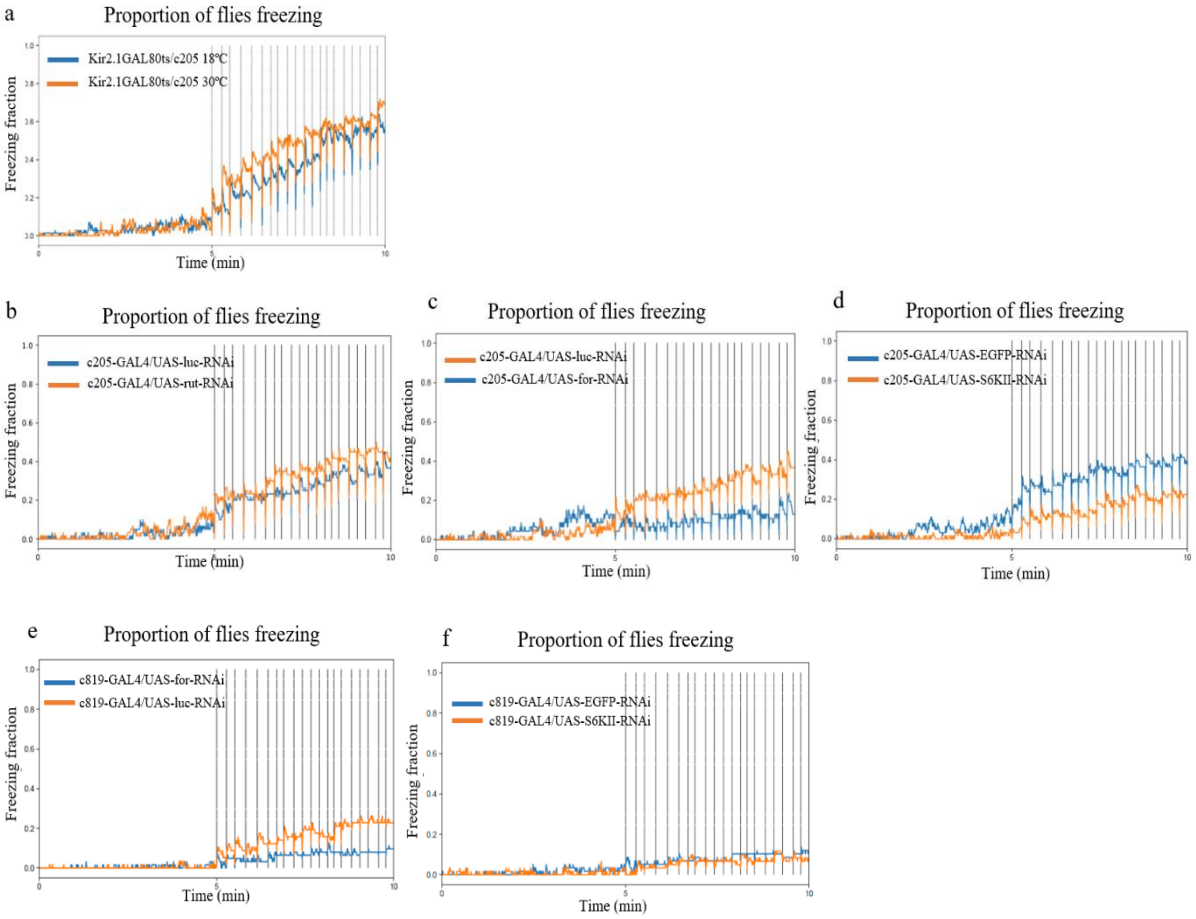
A6. Non-statistically significant results regarding the *for*S flies experiment (5 minutes baseline) and the walking speed parameters not mentioned in the main text. Walking speed during across the experiment. Dashed lines indicate stimulus presentations (a), walking speed during baseline (*for*^{S}/+: median = 11.7075 mm/s, IQR = [9.898; 13.2037]; *for*^{S}: median = 9.7268 mm/s, IQR = [8.876; 10.790]. Kruskal-Wallis test, $p < 0.001$) (b), walking speed during stimulation (*for*^{S}/+: median = 15.244 mm/s, IQR = [12.454; 16.546]; *for*^{S}: median = 13.05 mm/s, IQR = [11.98; 13.912]. Kruskal-Wallis test, $p < 0.001$) (c), change in walking speed caused by the looming presentation (baseline period subtracted from stimulation period) (*for*^{S}/+: median = 3.027%, IQR = [1.96; 4.486]; *for*^{S}: median = 2.823 mm/s, IQR = [2.360; 3.6015]. Kruskal Wallis test, $p = 0.7046$) (d), walking speed before the looming presentation (*for*^{S}/+: median = 13.57mm/s, IQR = [1.44; 15.675]; *for*^{S}: median = 12.3849 mm/s, IQR = [11.135; 13.425]. Kruskal-wallis test, $p < 0.05$) (e), change in walking speed caused by stimulus presentation (pre-stimulus period subtracted from post-stimulus period) (*for*^{S}/+: median = 4.5925 mm/s, IQR = [2.50; 7.91]; *for*^{S}: median = 3.36 mm/s, IQR = [1.83; 4.57]. Kruskal-wallis test, $p < 0.05$) (f), distribution of path orientations for both control and *for*-knockdown flies before and after looming walking trials. Bar height indicate counts (*for*^{S}/+: Mann-Whitney test, $p < 0.001$; *for*^{S}: Mann-Whitney test, $p < 0.001$) (g). * denotes $p<0.05$; *** denotes $p<0.01$; NS = not significant.

G. Freezing probability



A7. Freezing probability depending of the walking speed of the flies. *rut*-knockdown experiment (a), *for*-knockdown experiment (b), *S6KII*-knockdown experiment (c), *rut*-overexpression experiment (d), *rut* mutants experiment (5 minutes baseline) (e), *rut* mutants experiment (30 seconds baseline) (f), *for*S flies experiment (30 seconds baseline) (g), *for*S flies experiment (5 minutes baseline) (h).

H. Preliminary experiments



A8. Preliminary experiments. Freezing fraction of flies in which F5 neurons were silenced (a), freezing fraction of flies in which *rut* was knocked down in F5 neurons (b), freezing fraction of flies in which *for* was knocked down in F5 neurons (c), freezing fraction of flies in which *S6KII* was knocked down in F5 neurons (d), freezing fraction of flies in which *for* was knocked down in R3 and R4m neurons (e), freezing fraction of flies in which *S6KII* was knocked down in R3 and R4m neurons (f).