ISPA
INSTITUTO UNIVERSITÁRIO
CIÊNCIAS PSICOLÓGICAS, SOCIAIS E DA VIDA

Neuropeptides and the social modulation of defensive behaviors in drosophila

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Tese submetida como requisito parcial para a obtenção do grau de:

MESTRE EM NEUROCIÊNCIAS COGNITIVAS E COMPORTAMENTAIS

2017

Dissertação de Mestrado realizada sob a orientação da Doutora Marta Moita, apresentada no ISPA – Instituto Universitário para obtenção de grau de Mestre na especialidade de Neurociências Cognitivas e Comportamentais.

Agradecimentos

À doutora e colega Clara Ferreira, em especial, por me ter recebido e apoiado tão calorosamente no seu projeto. Um sincero obrigado por todos os conhecimentos, críticas e debates partilhados com os quais tanto aprendi ao longo deste ano. Por fim, desejo reconhecer a ajuda indispensável na preparação deste projeto e manuscrito.

À doutora Marta Moita, orientadora e mentora, por me ter recebido e apoiado neste projecto. Em empecial, agradeço os desafios colocados e votos de confiança que tanto enriqueceram esta experiência. Agradeço ainda a ajuda indispensável na preparação deste manuscrito.

A toda a equipa do *Moita Lab* — Andreia Cruz, Alexandra Medeiros, Clara Ferreira, Marta Moita, Matheus Silva, Mirjam Heinemans, Natalia Barrios, Ricardo Silva, Ricardo Zacarias e Scott Rennie — pelo suporte e companheirismo proporcionados ao longo deste ano de trabalho.

To Evelin Schmidt, dear partner and friend, for her valiant efforts to keep me fed, rested and possibly sane during these past few years; as well as for vital great help in the revision of this manuscript.

À Carolina Alves, colega de turma e amiga, por todo o apoio, paciência e companhia disponibilizados ao longo destes dois longos anos.

À minha família - e irmão em especial - assim como amigos mais próximos, por todas as coisas que, de uma forma ou outra, aqui me trouxeram.

Resumo

As moscas da fruta *Drosophila melanogaster* exibem longos períodos de imobilidade (*freezing*) em resposta a repetidos estímulos visuais representando um objeto em rota de colisão (*looming*), dos quais não podem escapar; comportamento este que é atenuado na presença de conspecíficos. No presente trabalho procedeu-se a um ensaio comportamental com mutantes de perda de função genética com o objectivo de identificar neuropéptidos e enzimas envolvidas na produção de neuropéptidos que possam estar envolvidos neste fenómeno de grupo. As moscas foram colocadas em arenas vedadas, sozinhas ou em grupos de 3 ou 5, e expostas a 20 estímulos *looming*. Identificaram-se duas linhas com fenótipos distintos: Amontillado e Phe-Met-Arg-Phe-NH_2 (FMRF). Os mutantes da enzima Amontillado demonstraram uma diminuição do efeito de grupo sobre o *freezing*, sendo que as moscas testadas em grupos exibiram níveis de *freezing* elevados e semelhantes aos de moscas testadas individualmente. Em contraste, os mutantes dos neuropeptidos FMRF demonstraram um efeito de grupo acentuado, com níveis de *freezing* substancialmente reduzidos nas moscas testadas em grupos. Quando as moscas foram testadas individualmente, ambas as linhas mutantes mostraram níveis de *freezing* equiparáveis aos das moscas controlo do tipo selvagem e controlo genético, sugerindo que estes fenótipos são específicos das condições de grupo. O fenótipo do mutante FMRF foi replicado através de um cruzamento entre esta linha e uma linha de deficiência com a região do gene FMRF removida. Este efeito não foi contudo observável com a expressão pan-neuronal de linhas RNA interferência para este gene, possivelmente devido a problemas destas linhas. Por fim, obtivemos dados preliminares de que moscas FMRF quando sozinhas também mostram respostas reduzidas de *freezing* face a estímulos looming menos intensos. Possivelmente, a combinação deste fenótipo individual com o efeito de *freezing* reduzido nos grupos de moscas de tipo selvagem, poderão explicar os baixos níveis de *freezing* dos grupos FMRF.

Palavras Chave: *Drosophila melanogaster*, Comportamento defensivo, Modulação social, Neuropéptidos, Amontillado, FMRF.

Abstract

Drosophila melanogaster fruit flies exhibit sustained immobility (freezing) in response to inescapable visual threats representing an object on collision course (looming); a behavior that is partially reduced when flies are in the presence of conspecifics. In the present work, we performed a behavioral loss of function screen to identify neuropeptides and neuropeptide processing molecules that might be involved in this group phenomenon. Flies were placed in an enclosed arena alone or in groups of 3 or 5, and exposed to 20 virtual looming stimuli. We identified two mutant lines with diverging group phenotypes: Amontillado and Phe-Met-Arg-Phe-NH_2 (FMRF). Amontillado enzyme mutants displayed a reduction of the group effect on freezing, with flies in groups freezing as much as flies alone. In contrast, FMRF neuropeptide mutants displayed a stronger group effect, with flies in groups showing substantially less freezing. When flies were tested alone, both mutant lines exhibited levels of freezing comparable to those of single wild-type flies and genetic background controls, suggesting these were group-specific phenotypes. We further replicated the FMRF phenotype by crossing the mutant line with a deficiency line in which the FMRF gene region is deleted, but failed to do so via pan-neuronal expression of RNA interference targeting this gene, possibly due to problems with the driver lines. Finally, we showed preliminary evidence suggesting that single FMRF flies also have a lower freezing response when looming stimuli are less intense. We hypothesize that the combination of this individual phenotype with the wild-type group effect towards less freezing could explain the floor levels of freezing observed in the FMRF groups.

Keywords: *Drosophila melanogaster*, Defensive behavior, Social modulation, Neuropeptides, Amontillado, FMRF.

Index

Figures Index

Tables Index

Introduction

The ability to recognize and evade fast-approaching physical and biological (i.e. predators) objects is essential for animal survival. This type of event produces a distinct visual pattern on the retina commonly referred to as a loom: a dark spot expanding outwards at an exponential rate. The link between loom detection and defensive responses was formally proposed by Gibson in 1958, and since then has been demonstrated in several species of insects, birds, fish, reptiles, crustaceans and mammals (Schiff, 1965; see reviews by Herberholz & Marquart, 2012; Pereira & Moita, 2016; Peek & Card, 2016).

Loom triggered defensive behaviors offer a good paradigm to study the neurological links between the acquisition of external biophysical information, decision making, and behavior output. The ubiquity of collision threats likely led to the evolution of common neural circuitry, while the speed and accuracy demands placed on these circuits suggest that they rely on few and large neurons (see Sterling & Laughlin, 2015). At the same time, these circuits should remain flexible enough to accommodate different strategies based on context (e.g., run if there is a shelter nearby, remain immobile otherwise; fly away if possible, run otherwise). These aspects make such circuits amenable to the use of comparative techniques as well as to their study in model organisms for which more refined tools are available. In the present work, we adopt this paradigm to assess the potential role of neuropeptide signaling on the social modulation of defensive behaviors in the fruit fly *Drosophila melanogaster*.

Responses to looming stimuli in *Drosophila melanogaster*

Recent work with this species has begun to elucidate some aspects of neural circuitry underlying the detection of looming stimuli and their complex behavioral responses. Work by Card & Dickinson (2008) showed that flies perform a series of behavioral adjustments prior to flight takeoff that direct them away from the source of a loom. Von Reyn and colleagues (2014) found that flies can opt between a fast takeoff jump with their wings lowered or a slower one with raised wings that improves flight stability, based on the spike timing of two circuits. Beyond this, flies are also capable of evading looming stimuli mid-air (Muijres et al., 2014).

Wu and colleagues (2016) showed that sometimes, instead of flight takeoff, flies respond to looming stimuli with backwards motion, a behavior that can also be elicited by activating a subset of visual projecting neurons. Two independent studies (Gibson et al., 2016; Zacarias et al., unpublished) showed that flies placed in enclosed arenas can shift their responses to include running or freezing, with the latter group having identified a pair of descending fibers that mediate freezing. Importantly, all these behaviors have been observed in *Drosophila* interactions with natural predators (Parigi et al., 2014).

Flies' responses can also scale with repeated inescapable looming stimuli, in terms of increased walking speed or higher likelihoods of jumping or remaining completely immobile (i.e., freezing; Gibson et al., 2016; Zacarias et al., unpublished). Interestingly, work from our lab (Ferreira & Moita, unpublished) showed that the freezing response can be modulated by the number of same-sex conspecifics present in the arena. Specifically, they revealed that flies are more likely to break from this state as the number of conspecifics increases.

Social modulation of defensive behaviors

Social modulation of defensive behaviors has been previously reported in *Drosophila*. Kacsoh and colleagues (2015) demonstrated a mechanism of visual social transmission whereby flies suppress oviposition after encountering egg-eating wasps or interacting with other flies that had encountered them recently. Flies also seem capable of simple forms of collective behavior, such as displaying a more vigorous escape from an aversive odor when in the presence of other conspecifics (Ramdya et al., 2015). Another study on flight performance during prey-predator interactions suggested that the number of conspecifics with whom a fly has recently interacted could affect its subsequent escape trajectories, making them more or less erratic (Combe et al., 2012). These studies offer tentative evidence that in some contexts the fruit fly is able to incorporate recent information about its social context to tune specific defensive behaviors.

This leads to the intriguing question of how such processes could be encoded at the neural level. One hypothesis that is derived from research on higher order animals involves neuropeptide modulation. Research on social buffering in mammals — a phenomenon whereby the presence of conspecifics attenuates the negative effects of aversive experiences — has suggested a central role of the oxytocin neuropeptide (see reviews by Kikusui et al., 2006; Hennessy et al., 2009; Hostinar et al., 2014).

Repeated positive interactions with conspecifics (e.g., parental caring and peer grooming) can lead to the release of oxytocin in the Central Nervous System (CNS) with both short- and long-term effects over stressful situations. In a human experiment by Heinrichs and colleagues (2003), nasal administration of oxytocin reduced the levels of cortisol and behavioral manifestations of stress during a social anxiety task, to the same extent as receiving support from a close friend. Similarly, Windle and colleagues (2004) showed that central injections of oxytocin reduce the levels of corticotropin release hormone mRNA after a 30-minute restraint session in rats. Recently, Rickenbacher and colleagues (2017) demonstrated that oxytocin in the CNS mediates the transition from a mother's self-defense to pup protection. At larger time frames, Winslow and colleagues (2003) showed that rhesus monkeys reared alone by humans have lower basal levels of oxytocin compared to peers reared by their mothers, which might later condition their ability to interact or be calmed by the presence of peers; furthermore, Danilowski and colleagues (2016) drew a link between oxytocin and the long-term neural effects of childhood trauma in humans.

Although similar neuromodulatory mechanisms underlying the social modulation of defensive behaviors are yet to be described in *Drosophila melanogaster*, they have been implicated in other complex social behaviors of this species. Different aspects of male sexual and aggressive behaviors have been shown to be modulated by very small subsets of neurons that release octopamine (Certel et al., 2013), serotonin (Alekseyenko et al., 2014), dopamine (Alekseyenko et al., 2013), insulin (Luo et al., 2014) and tachykinin (Asahina, 2014).

Present work

The goal of the present work was to conduct a behavioral screen of neuropeptide and neuropeptide processing mutants that might be involved in the social modulation of *Drosophila*'s responses to repeated inescapable looming stimuli, and more specifically that of freezing.

According to a review by Nässel and Winther (2010), there are 42 genes in the *Drosophila* genome that code for precursors of neuropeptides, peptide hormones and protein hormones. Computer analysis suggests there might be up to 75 viable peptides in *Drosophila*, although some are likely to be redundant or inert. The most extensive empirical assay of the neuropeptides in the adult fly identified 48 peptides originating from 18 genes (Yew et al., 2009).

Due to logistical constraints, we prioritized a set of candidate neuropeptide genes based on the literature available. A molecular homolog to oxytocin — inotocin — has been described in several arthropods, but is absent in *Drosophila melanogaster* (Stafflinger et al., 2008). However, since inotocin has been found to exert diuretic effects, we looked at neuropeptides with similar functions in the *Drosophila* such as the diuretic hormone 44 (DH44; other peptides with similar functions include the DH33, leucokinin, and CAPA; Nässel & Winther, 2010). The involvement of crustacean cardioactive peptide (CCAP) was also tested based on its structural similarities to oxytocin and vasopressin (Nässel & Winther, 2010). We selected the Phe-Met-Arg-Phe-NH_2 (FMRF) gene due to its roles in escape responses by larvae (Klose et al., 2010) and adults (Kiss et al., 2013).

We also looked at neuropeptide processing molecules, as they could offer a strong evidence for the involvement of neuropeptide modulation in the social modulation of defensive responses. Here we focused on the Amontillado enzyme, the homolog of the mammal PC2 which plays a critical role in the early biosynthesis of most if not all neuropeptides in *Drosophila* (Wegener et al., 2011). If Amontillado were to have any observable effect, we would then include the remaining neuropeptides that have been confirmed to be processed by this enzyme such as Myosuppressin (Rhea et al., 2010; Wegener et al., 2011).

We compared the behaviors of flies tested alone with those in groups of 3 and 5, to identify mutants with either an increased or dampened group modulation over freezing. For control purposes, we matched the genetic mutants with the respective genetic background lines and wild-type flies. In addition, we planned two approaches in order to confirm the most promising hits we got from our mutant screen: a) complementation test of mutants crossed with a deficiency line and b) the use of RNA interference coupled to a neuronal Gal4 driver. At a final stage, we ran a set of experiments in varying looming intensity to assess whether the mutant phenotypes were restricted to the group conditions or if they also affected the behavior of flies tested alone.

Methods

Fly husbandry

Flies were kept on standard medium in low density conditions (groups of 20 females and 8 males) under a 12-hour light/dark cycle. They were flipped every two or three days to prevent overcrowding of larvae, and after two or three weeks egg-laying adults were replaced with younger flies. Flies were kept at 25 °C (70% humidity), except for short periods of overnight cooling (18 °C) to facilitate collection of virgins for crosses.

To ensure that flies were 5 to 7 days old at the time of experiment and to prevent any side effects of anesthesia (CO2), the flies were transferred to vials containing the same medium two days after eclosion started and tested only 4 to 5 days later. The flies were kept in groups of 27-32 with a 3:1 sex ratio to ensure mating. Annex A contains the listing of all the strains used.

Deficiency lines and Gal-4 x RNAi UAS

To confirm that any hits from our mutant screen were actually due to the impairment of the genes of interest, we planned to use two complementary methods: crossing mutants with deficiency lines in which the gene region is deleted, and expressing RNA interference with the use of the Gal4 -UAS dual expression system.

Deficiency lines with defined chromosomal deletions are often used in *Drosophila* genetics to confirm that a given phenotype is due to a recessive mutation in the gene of interest (Cook et al., 2012). This is done via a simple complementation test, where the two lines are crossed and the persistence of the mutant phenotype in offspring can, for most cases, be taken as evidence for the role of the gene. If, on the other hand, the offspring show a wild-type phenotype, this usually means that the original mutant phenotype was related to other affected genes beyond the deleted region (fig. i; McClean, 1999; Fay, 2006; Yook, 2005).

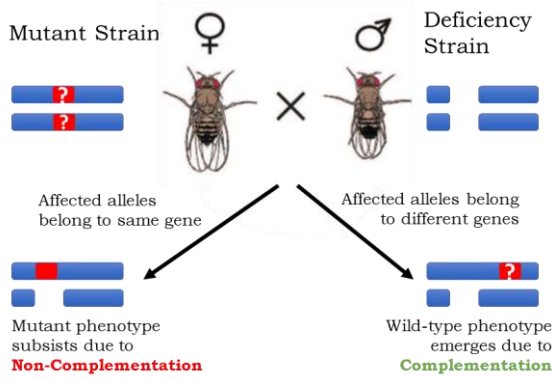


Figure i. Schematic of a complementation test using a deficiency line. If the deleted portion of DNA in the deficiency line includes the mutated gene of the mutant strain, offspring will most likely exhibit the mutant phenotype. If the mutated gene is not inside the deleted section, offspring will most likely exhibit wild-type phenotype due to allele complementation. This assumes that the mutation is recessive.

RNA interference (RNAi) suppresses gene activity by exploiting the RNA-induced silencing complex (RISC) to target and neutralize specific mRNA molecules (Dietzl et al., 2007). The Gal4/UAS system constrains the expression of an inserted gene to specific cells by pairing it to the UAS construct, which acts as a switch to gene expression based on the presence or absence of the yeast transcription activator protein that is encoded by the Gal4 gene (fig ii; Brand & Perrimon, 1993; Duffy, 2002). We used two lines that drive expression on neuronal tissues: Nsyb and elav (Estes et al., 2000; Lin & Goodman, 1994).

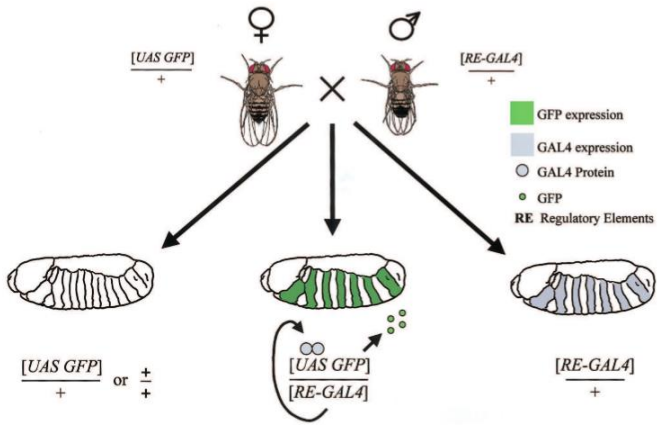


Figure ii. Schematic of the bipartite UAS/GAL4 system in Drosophila. "When females carrying a UAS responder (UAS-GFP) are mated to males carrying a GAL4 driver progeny containing both elements of the system are produced. The presence of GAL4 in an alternating segmental pattern in the depicted embryos then drives expression of the UAS responder gene in a corresponding pattern" (Duffy et al., 2002). In the present work, this system was used to express RNA interference on neuronal tissues. Image source: Duffy et al., 2002

Behavioral experiments

The behavioral setup used in this work was adapted by Ferreira and Moita (unpublished) from the original design by Zacarias and colleagues (unpublished) to allow the study of group and single flies' responses to repeated inescapable looming stimuli. The flies were tested alone or in groups of 3 and 5, in round chambers (68 mm radius, 3.5 mm high; fig. iii left; see annex B for detailed specifications) covered by a glass panel. The arenas were retro illuminated by infrared LEDs, and two cameras placed directly above recorded the flies at 60fps (fig. iii, right). A filter for visible wavelengths was used to reduce visual noise. The stimuli were delivered by a monitor (24", 144Hz), placed 20 cm away from the arenas at a 45° angle. An infrared LED linked to an Arduino circuit blinked at the start and end of the experiment and onset of looming stimuli, to facilitate the alignment of the videos during analysis. The entire setup was enclosed in an opaque black box, in a room with the same conditions of rearing.

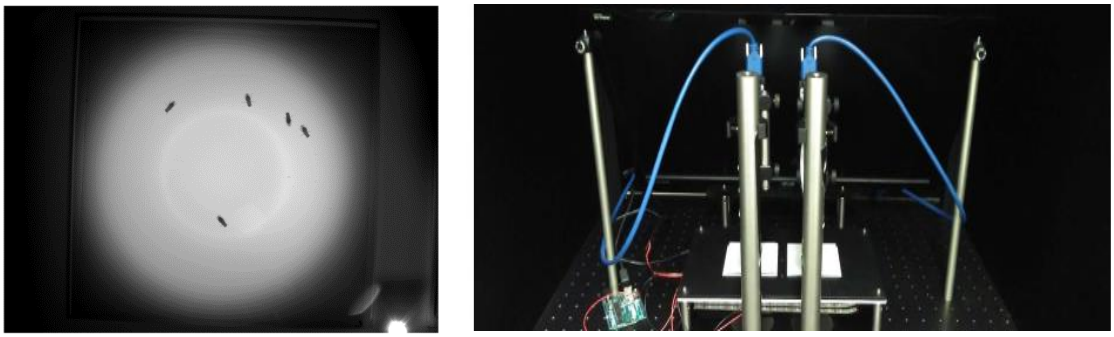


Figure iii. Fly chambers (left) and experimental setup (right).

The behavioral protocol was created in PsychoPy (Peirce, 2007) and included a 5-minute baseline period, during which the monitor screen was kept white, followed by a 5-minute stimulation period where the flies were exposed to 20 looming stimuli distributed quasi-randomly, with an inter-stimulus interval ranging from 10 to 20 seconds (fig. iv). The looming stimuli consisted of quickly expanding 100% solid black circles, except for the last set of experiments in which we changed them to 27%, 9% and 3% black, in order to reduce their intensity. The visual angle (θ) of the circle at each frame was calculated from the following formula:

$$\theta(t) = 2 \times \tan^{-1}(l \div (v \times t))$$

Where t is the time to collision (seconds), l is half of the length of the object (cm) and v the velocity of the object towards the fly (cm/s). We set l to 1 and v to 25, to simulate the visual dynamics of an object with 1 cm radius that is approaching at a constant velocity of 25 cm/s. The looming stimuli appeared on the screen 500 ms before collision ($\theta = 9°$) and expanded until 50 ms before collision ($\theta = 72°$), after which they remained stationary for another 50 ms before disappearing. The final size reflected the largest circle that could still fit inside the monitor.

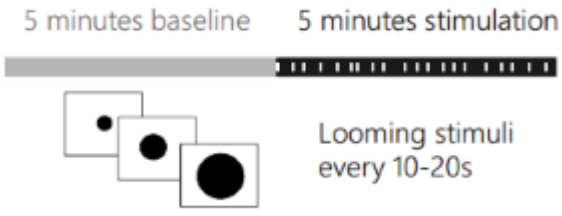


Figure iv. Behavioral protocol.

Behavioral analysis

The videos obtained were analyzed with idTracker (Pérez-Escudero et al., 2014) in order to identify individual flies and extract their respective trajectories, and then further processed by custom software (FlyMotion Quantifier, Champalimaud Software Platform) to calculate the speed and the values of pixel change around each fly (in a 50-pixel radius), as well as to estimate collisions between individuals. All further data processing was done with custom Python scripts. Speed measures were converted from pixel/frame to mm/s. To identify jumps, each fly's speed was parsed into a peak detection function based on changes in amplitude (Duarte, 2014; parameters: minimum peak height = 80, minimum peak distance = 10), and each peak was considered a jump. To extract the remaining behaviors, the speed data was first summarized into bins of 30 frames (0.5s, total of 1200 bins), using both the average and median functions. Pixel change was similarly summarized with a median function. Those bins with an average speed larger than 2.2 mm/s and a median speed larger than 1 mm/s were classified as walking, and the remaining classified as grooming if the median pixel change was larger than 30 or as freezing otherwise. A manual validation of the accuracy of these estimations can be found in the Annex C.

Statistical analysis

Since most behaviors did not follow a normal distribution, we chose to use nonparametric tests in all cases. We were not very concerned with a loss of power as our samples and main effects were relatively large. Quantitative group differences were analyzed with Kruskal-Wallis test, and Dunn post-hoc test adapted from a custom script (Muldal, 2014; slightly modified to accommodate correction for ties). The chi-square test was used to compare the binomial distribution of freezing at the last bin. Bonferroni correction was always applied during multiple comparisons. Paired differences from zero were computed with the Wilcoxon signed-rank test, after confirming that the distribution was symmetric. Unless otherwise specified, tests belonged to the SciPy library. Plots were created with the Python Matplotlib library.

Results

The experiments reported in this work were planned and analyzed in close collaboration with Clara Ferreira. The first two sections detail the behaviors of wild-type flies tested alone and how they compare with those of flies tested in groups. Based on previous work from our lab we expected to observe a robust reduction in the freezing response of flies as the number of conspecifics present in the arena increased, whereas other defensive behaviors (running and jumping) should remain mostly unaffected by the group context. The phenotype of wild-type flies was taken as one of the main controls (the other being the genetic background) for the phenotypes of the mutant flies described in the ensuing sections. To enable multiple comparisons, the experiments of wild-type, mutant, and genetic background lines were conducted in quick succession during a two-month period.

Wild-type flies

Wild-type flies display a strong freezing phenotype when tested alone

Figure 1 summarizes the observed behaviors of wild-type flies (Canton - S) tested alone. During baseline, flies sustained an intermediate level of activity, alternating between bouts of walking and grooming. They rarely paused or attempted takeoff by jumping, and the few jumps observed tended to be concentrated in the first half of the baseline (fig. 1A), suggesting a gradual habituation to the arena. Flies never entered in a freezing state — sustained immobility — during this period (although short pauses were sometimes categorized as such by our automatic behavior classification; see annex C).

Flies' behaviors changed drastically with the onset of looming presentations (stimulation period); the most remarkable being a steady increase in the proportion of flies freezing (fig. 1A). Males spent a median 71.7% of the stimulation time freezing (215 s) and females 40.3% (121 s). This was done mostly in a few large uninterrupted chunks, as evidenced by the fact that the median freezing bout lasted only 2.5 seconds for both sexes and that males had a median of 12 separate bouts and females half as much. Importantly, the increase in time spent freezing was not accompanied by an increase in time spent grooming (males: mdn = - 19 s, iqr = [-35.5; - 6], $p <$

0.001; females: mdn = 0 s, iqr = [-8; - 4], p = 0.22; Wilcoxon test), suggesting that the observed changes were not due to a simple cessation of walking behaviors.

Previous work (Gibson et al., 2016; Zacarias et al., unpublished) has shown that flies that are not freezing show an increase in walking speed. While we failed to see an increase in the average walking speed from baseline to stimulation (fig. 1B; males decrease: mdn = - 0.27 mm/s, iqr = [-2.23; 0.95], p = 0.02; females remain unchanged: mdn = 0.13 mm/s, iqr = [-0.47; 0.96], p = 0.14; Wilcoxon test), we observed transient peaks when comparing the first second before and after each looming (change after looming; fig. 1C; males: p < 0.001; females: p = 0.002; Wilcoxon test). These changes were more modest than we had anticipated, and might be explained by differences in how flies navigate our arenas. In our experiments, flies keep running towards the perimeter of the arena where they stop to turn, before repeating again; whereas they tend to run continuously in the arenas used previously in our lab (Zacarias et al., unpublished).

As reported in previous studies (Gibson et al., 2016; Zacarias et al., unpublished), we also observed jumps in response to repeated inescapable looming (fig. 1A). Both sexes displayed more jumps during stimulation than baseline (males: mdn increase = 2, iqr = [1; 5], p < 0.001; females: mdn increase = 1, iqr = [0; 2] p < 0.001; Wilcoxon test), with 62% of these happening in the first second after a looming and 84% in the first 5 seconds. The fraction of males jumping declined during stimulation, from over 20% in the first looming bins to less than 10% by the end. A similar pattern for females is difficult to assess due to the low number of jumps observed.

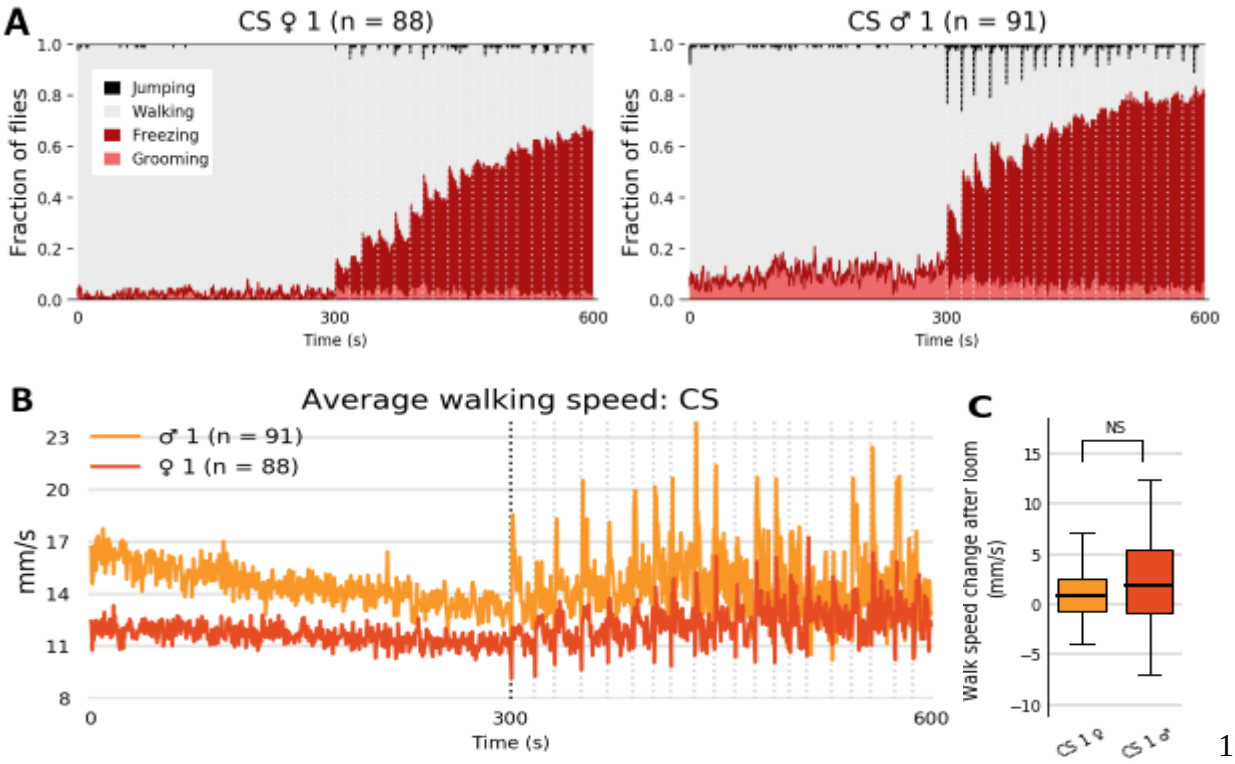


Figure 1. Wild-type flies tested alone. **A:** Cumulative proportion of flies freezing (red), grooming (pink), jumping (black) or walking (grey) across the experiment. **B:** Average walking speed across the experiment. **C:** Average change in walking speed around looming (1s after – 1s before; dashed median indicates nonsignificant difference from zero, $p > 0.05$, Kruskal-Wallis test). Vertical dashed lines indicate looming events. Females are shown in orange and males in yellow. NS indicates nonsignificant differences. Sample sizes $\subset [88, 91]$.

Overall, females and males showed similar behavioral patterns throughout the experiment, although males had consistently stronger responses, a trend that generally held in all subsequent experiments. Because the source and role of sexual differences in responses to looming lie beyond the scope of this work, they will not be discussed in further detail.

Wild-type flies display a dampened freezing phenotype when tested in groups

As previously observed in work from our lab, when flies were tested in groups of 3 and 5, there was a substantial reduction in the proportion of flies freezing (fig. 2A-B) and time spent freezing (fig. 2C-D). This effect was 'dose-dependent' as flies in groups of 5 showed a stronger shift than those in groups of 3. The probability of a fly freezing by the end of the experiment was different between the three conditions in both sexes ($p \leq 0.02$, Chi-square test), as was the time spent freezing ($p \leq 0.02$, Dunn test), with the exception of single females and those in groups of 3 ($p = 0.87$, Dunn test).

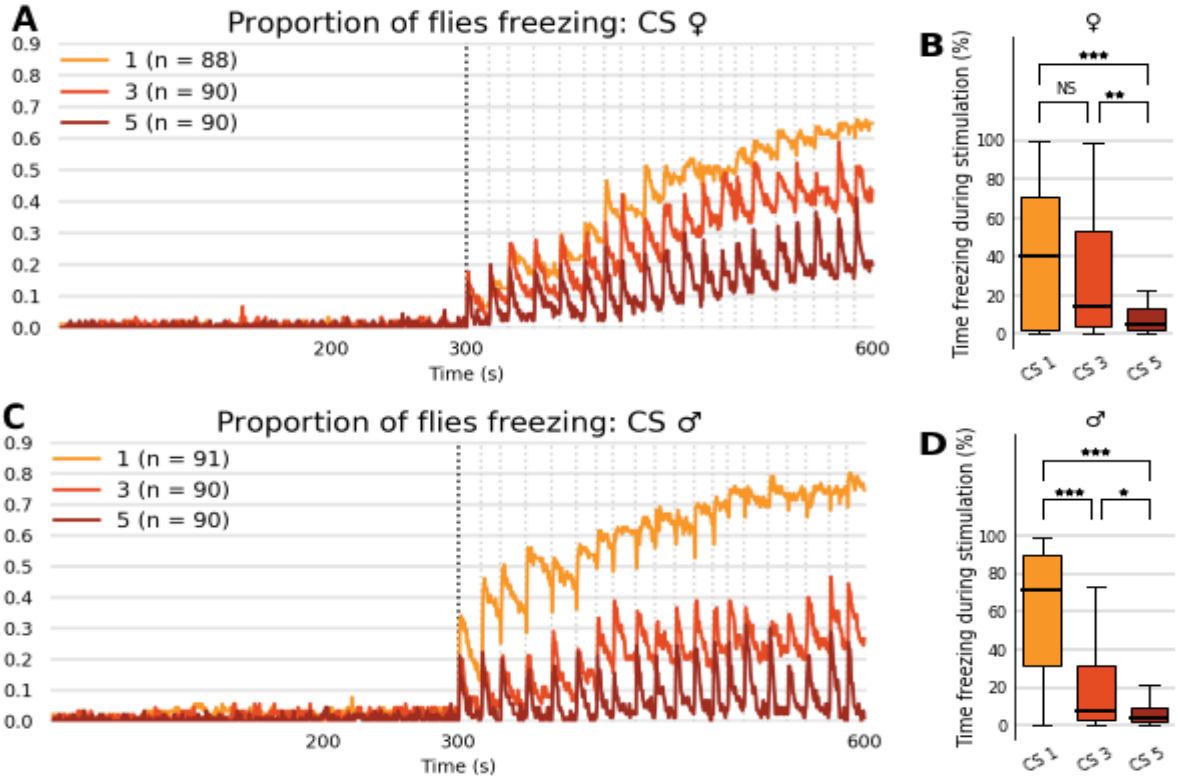


Figure 2. Wild-type flies — group comparisons regarding freezing. **A-C:** Proportion of flies freezing across the experiment. Vertical dashed lines indicate looming events. **B-D:** Percentage of time spent freezing during stimulation. Single flies are shown in yellow, groups of 3 in orange and groups of 5 in brown. Asterisks indicate significant pairwise differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$). NS indicates nonsignificant differences. Sample sizes $\subset [88, 91]$.

These group differences seem to reflect a tendency towards exiting sooner from freezing states, and not a weaker tendency to initiate freezing. There were no significant differences across male group conditions in the median number of freezing events (Ind = 12, G3 = 13, G5 = 10; $p = 0.30$, Kruskal-Wallis test), whereas female groups even exhibited a higher number of events relative to flies tested alone (Ind = 6, G3 = 10, G5 = 12; $p < 0.001$; Kruskal-Wallis test).

We hypothesized that the group reduction in freezing could be due to collisions between flies. Females in groups of 5 had a median of 30% of freezing offset events being immediately preceded by a collision with another animal (in groups of 3 this was 22%). However, we did not find a negative association between the proportion of freezing offset by collisions and total time spent freezing, as would be predicted by this hypothesis. Instead, there was a positive trend in the other direction, with flies that froze for longer periods of time having a higher proportion of freezing offset by collisions (fig 3; G3: $r = 0.04$, $p = 0.74$; G5: $r = 0.33$, $p = 0.001$; Pearson coefficient). We could also observe several instances in which flies sustained freezing even after a collision with another animal (data not quantified), and similar results were obtained for males (not shown for sake of brevity). Taken together, these analyses suggest that the earlier withdrawal from freezing when in groups reflects an active response by the flies. Ongoing work in the lab aims at understanding which cues underlie this phenomenon.

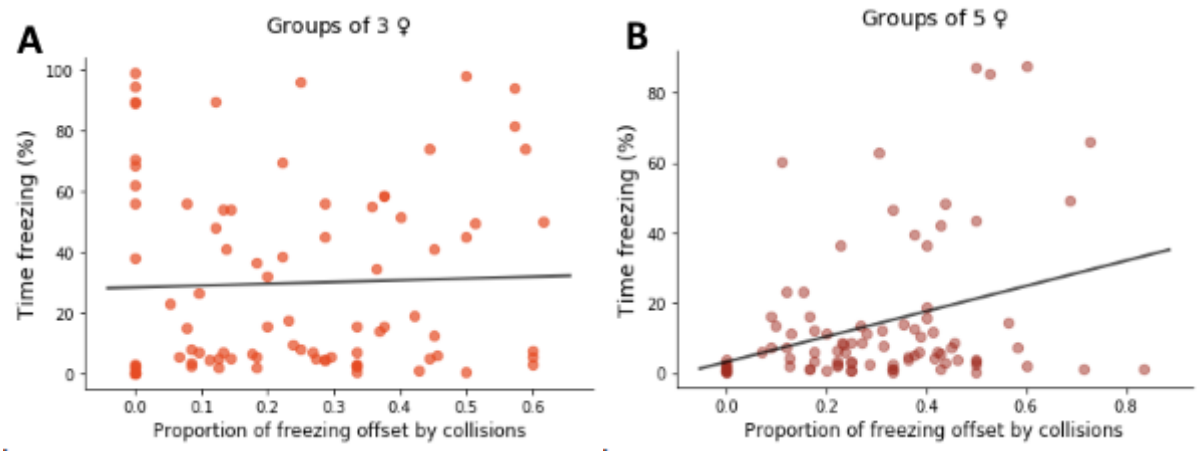


Figure 3. Female wild-type flies — Time spent freezing as a function of the proportion of freezing offset events caused by collisions. Black line represents the linear regression. **A:** Flies in groups of 3. **B:** Flies in groups of 5. Sample sizes = 90.

Other behaviors were less altered by the social context. Figures 4A,C show the average walking speed across the experiment for all group conditions. We chose to analyze the average change in speed around looming because it was the most robust measure in wild-type flies tested alone. Only males in groups of 3 showed a significant increase in this measure (fig. 4D, $p = 0.01$, Wilcoxon test), with flies in the remaining group conditions displaying a nonsignificant trend in the same direction (fig. 4B,D; $p \geq 0.07$, Wilcoxon test). There was a trend towards a group effect with larger groups exhibiting weaker responses, although the only significant differences detected were between male individual flies and those in groups of 5 ($p = 0.005$, Dunn test). Together with their reduced freezing phenotype, these results could be indicative of a lower arousal among flies tested in groups.

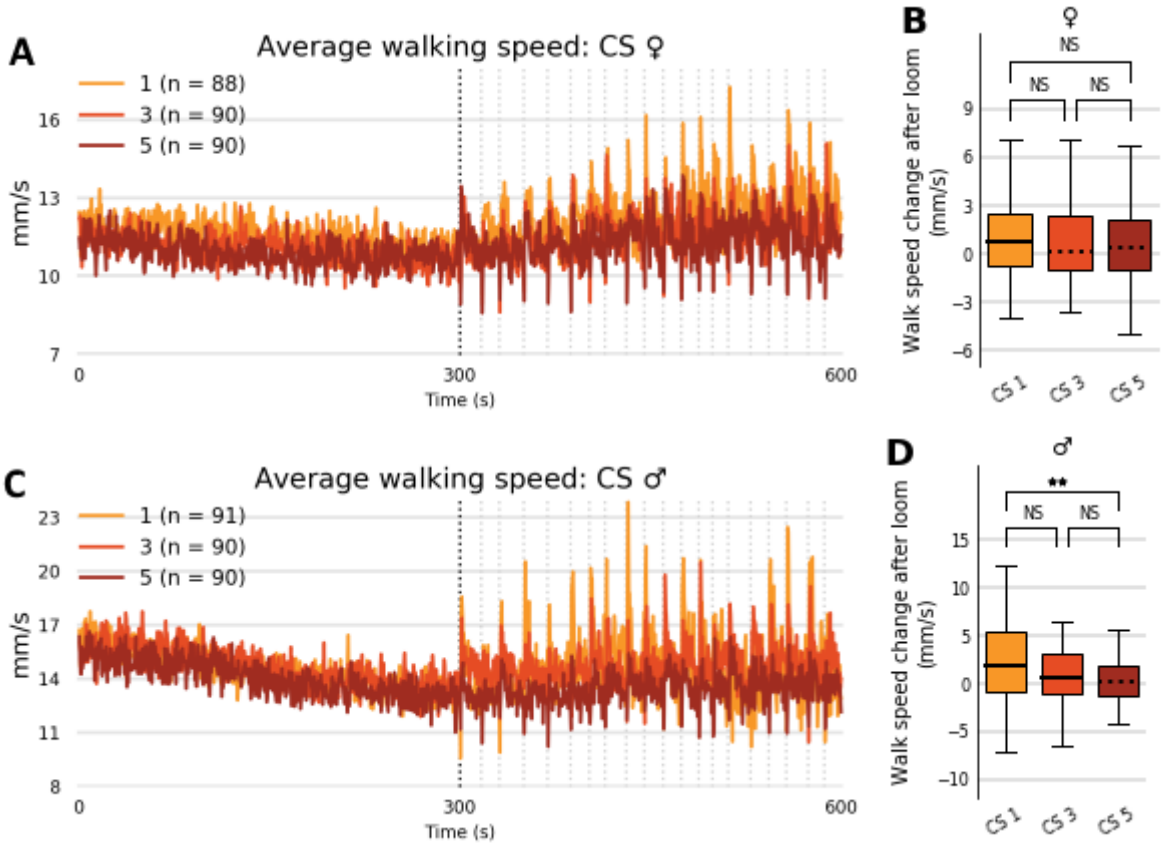


Figure 4. Wild-type flies — group comparisons regarding walking speed. **A, C:** Average walking speed across the experiment. Vertical dashed lines indicate looming events. **B, D:** Average change in walking speed around looming (1s after – 1s before; dashed median indicates nonsignificant difference from zero, $p > 0.05$, Kruskal-Wallis test). Females are shown on top and Males on bottom. Single flies are shown in yellow, groups of 3 in orange and groups of 5 in brown. Asterisks indicate significant pairwise differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$). NS indicates nonsignificant differences. Sample sizes $\subset [88, 91]$.

There was a significant group effect in jumps among males, with groups of 3 showing a higher number of jumps during stimulation (normalized by baseline, fig. 5B) than individual flies ($p = 0.007$, Dunn test) and a similar trend when compared to groups of 5 ($p = 0.051$, Dunn test). Among females, there were no significant differences across groups (fig. 5A; $p = 0.97$; Kruskal-Wallis test). Finally, we saw a higher drop in time spent grooming from baseline to stimulation among male flies tested alone compared to those in groups (fig. 5D, $p \leq 0.003$, Dunn test), but not among females (fig. 5C; $p = 0.12$, Kruskal-Wallis test). It is worth remembering, however, that our settings capture a considerable rate of false positive grooming events during stimulation (see annex C).

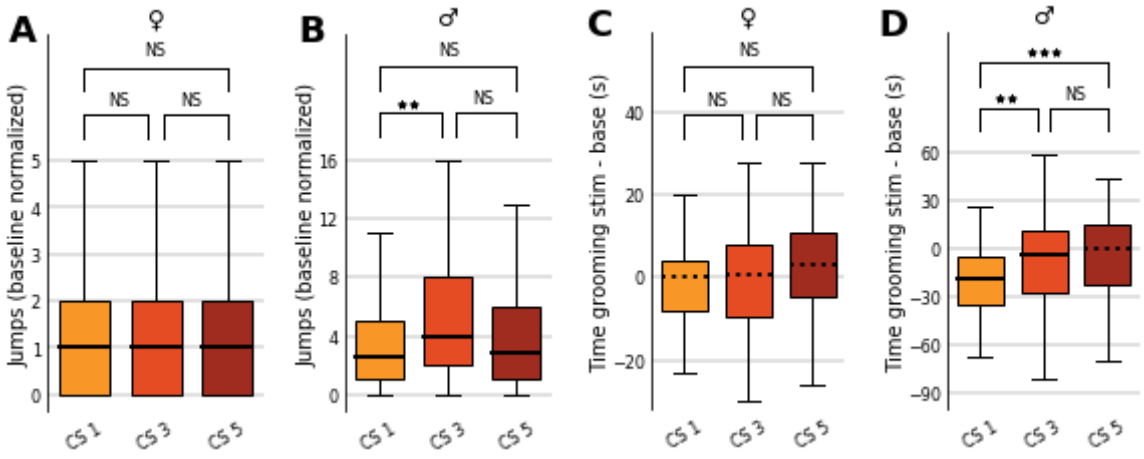


Figure 5. Wild-type flies — group comparisons regarding jumps and grooming. **A-B**: Jumps during stimulation normalized by each flies' baseline. **C-D**: Difference between time spent grooming in stimulation and baseline (dashed median indicates nonsignificant difference from zero, $p > 0.05$, Kruskal-Wallis test). Females are shown on the left and males on the right. Single flies are shown in yellow, groups of 3 in orange and groups of 5 in brown. Asterisks indicate significant pairwise differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$). NS indicates nonsignificant differences. Sample sizes $\subset [88, 91]$.

In summary, we found that wild-type flies tested in groups spent less time freezing and were less likely to be freezing by the end of the experiment when compared to flies tested alone. This seems to reflect a tendency to exit sooner from freezing, since flies in groups did not exhibit a lower number of freezing events. There was no indication of a shift towards other defensive responses among flies tested in groups, as the number of jumps and the magnitude of change in walking velocity was comparable or even inferior to those of individual flies. Having replicated and characterized the phenotype of wild-type flies, we then proceeded to test the effects of neuropeptide and neuropeptide processing mutant strains on group behavior.

Neuropeptide and neuropeptide processing mutants

Four neuropeptide mutant lines — Phe-Met-Arg-Phe-NH_2 (FMRF), Crustacean Cardioactive Peptide (CCAP), Diuretic Hormone 44 (DH44) and Myosuppressin (Ms) — and two mutant lines for the neuropeptide processing Amontillado enzyme were selected based on the available literature. In order to identify phenotypes specific to the social conditions, we excluded those strains in which single flies of both sexes displayed weak patterns of freezing. Figure 6 shows the percentage of time spent freezing during stimulation of mutant flies tested alone compared to wild-type flies. We observed a strongly reduced response by both DH44 and Amontillado B (Mi{ET1}amon^{MB04710}) males and females, as well as by Ms females (Ms experiments were done at a separate time and males were not tested). Visual inspection of jumps and walking speed plots also revealed little modulation to looming stimuli. Possibly related, the first two lines share the same type of mutation (minos insertion) and genetic background (w^{1118}) which is known to display several visual and behavioral abnormalities (Bulgakova et al., 2010; Sitaraman et al., 2008; Liu et al., 2006). Since the flies tested in groups did not show strong responses either, we discarded these three lines for being unresponsive.

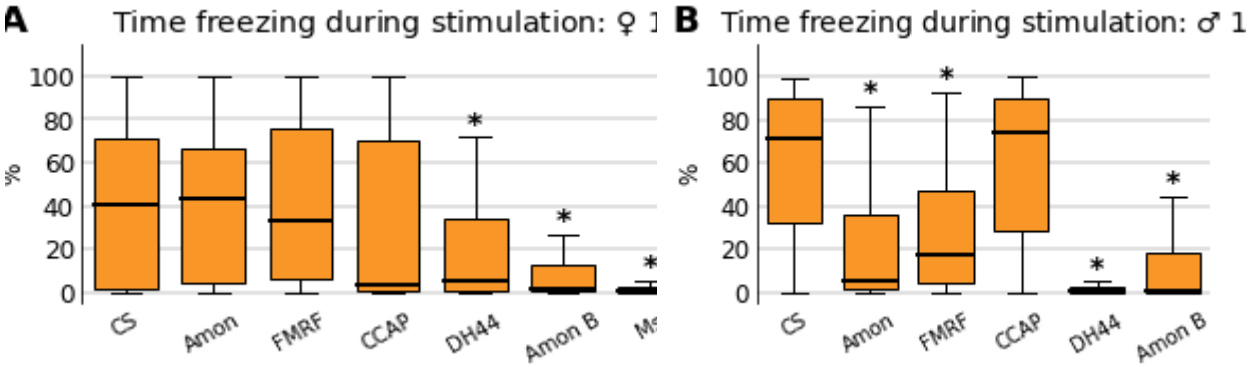


Figure 6. Comparison between wild-type flies tested alone and mutant strains, regarding percentage of time spent freezing during stimulation. Amontillado (Amon) A and B (respectively, Mi{ET1}amon^{MB00756} and Mi{ET1}amon^{MB04710}), Phe-Met-Arg-Phe-NH_2 (FMRF), Crustacean cardioactive peptide (CCAP), Diuretic Hormone 44 (DH44) and Myosuppressin (MS). Asterisks indicate significant differences from wild-type flies ($p \leq 0.05$). **A:** Females. **B:** Males. Sample sizes $\subset$ [88, 102] for CS, Amon A and FMRF; and sample sizes $\subset$ [36, 51] for the remaining strains.

The next sections focus on the individual and group phenotypes of Amontillado and FMRF mutants. These results contain data from two independent experiments that were merged after confirming its similitude (analysis not shown). Data from CCAP mutants, which exhibited a more ambiguous phenotype, can be found in Annex D.

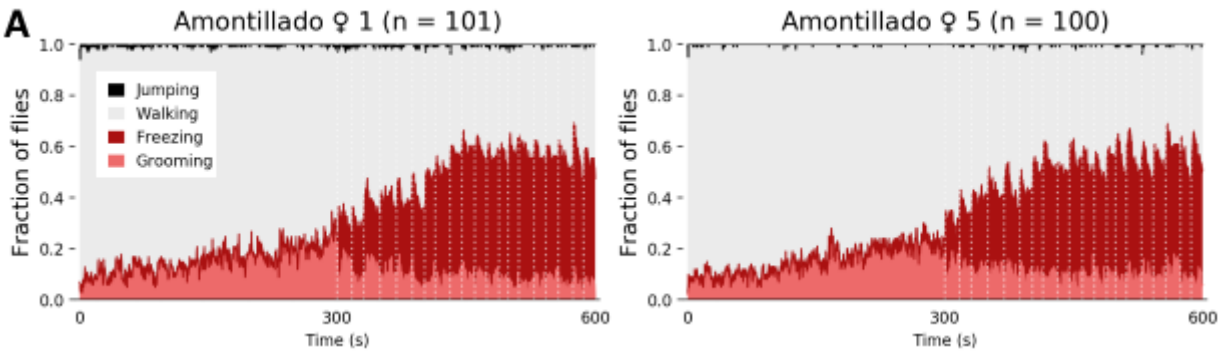
The freezing group effect is suppressed in Amontillado mutants

Figure 7 depicts the behaviors of Amontillado mutant flies (Mi{ET1}amon^{MB00756}). Neither sex exhibited a group effect on freezing, regarding the final proportion of flies freezing (fig. 7B,D; females: $p = 0.96$; males: $p = 0.12$, Chi-square test) or time spent freezing (fig. 7C,E; females: $p = 0.96$; males: $p = 0.98$; Kruskal-Wallis test). However, male flies tested alone showed substantially less freezing compared to wild-type flies, a phenotype that might be related to the background line and not to the Amontillado mutation (see background control section).

Amontillado mutants displayed a gradual reduction in walking speed, starting at baseline, that continued during stimulation (fig. 7F, H), with all group conditions of both sexes reducing their average speed from baseline to stimulation by about 2 mm/s ($p < 0.001$, Wilcoxon test). Also, with the exception of females tested alone, all groups exhibited a mean reduction in walking speed around looming, although this was not always significant (fig. 7G, I). This parameter was not significantly different between group conditions ($p \geq 0.12$, Kruskal-Wallis test).

Finally, Amontillado mutants displayed few jumps, most of which were not temporally correlated with looming, as less than 50% of the stimulation jumps occurred in the 5 seconds following a looming. Single flies tended to jump slightly more than groups (fig. 7J-K).

These results confirmed the potential of our strategy to target the social modulatory effects of defensive responses to looming, while supporting an involvement of neuropeptide signaling. Since the Amontillado enzyme acts early in the biosynthetic pathway of the majority of *Drosophila*'s neuropeptides, it is plausible that the phenotype observed is due to changes in the functioning of a more limited range of downstream neuropeptides.



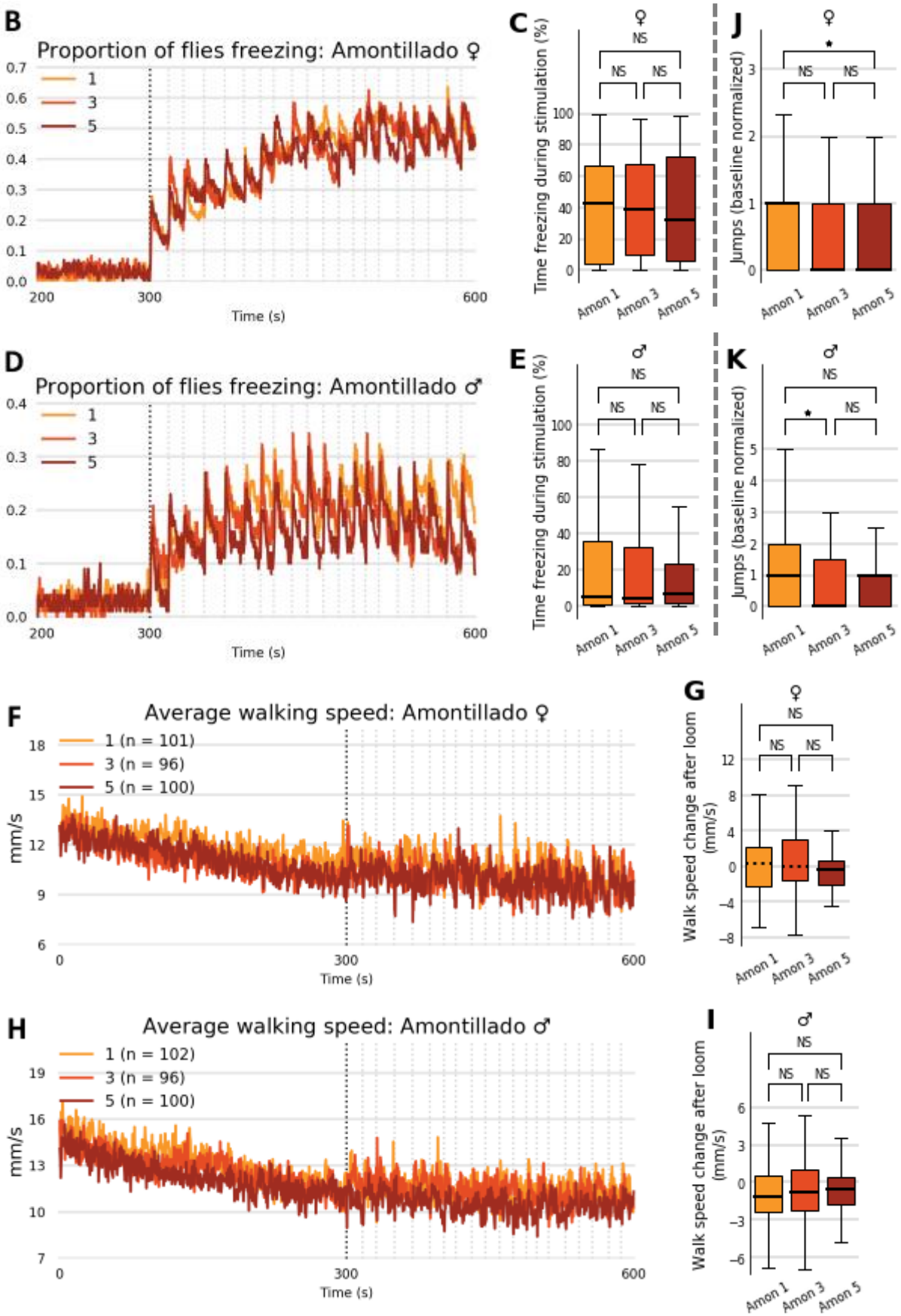

B
Proportion of flies freezing: Amontillado ♀
1
3
5
Time (s)
C
♀
Time freezing during stimulation (%)
NS
Amon 1
Amon 3
Amon 5
J
Jumps (baseline normalized)
*
D
Proportion of flies freezing: Amontillado ♂
E
♂
K
F
Average walking speed: Amontillado ♀
1 (n = 101)
3 (n = 96)
5 (n = 100)
mm/s
G
Walk speed change after loom (mm/s)
H
Average walking speed: Amontillado ♂
1 (n = 102)
3 (n = 96)
5 (n = 100)
I

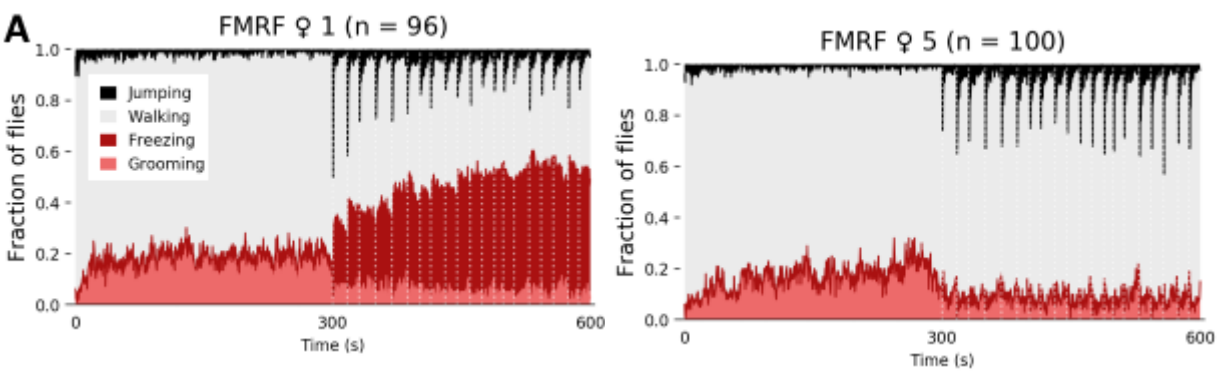
Figure 7. Amontillado mutants. **A:** Cumulative proportion of flies freezing (red), grooming (pink), jumping (black) or walking (grey) across the experiment. **B, D:** Proportion of flies freezing across the experiment. **C, E:** Percentage of time spent freezing during stimulation. **F, H:** Average walking speed across the experiment. **G, I:** Average change in walking speed around looming (1s after – 1s before; dashed median indicates nonsignificant difference from zero, $p > 0.05$, Kruskal-Wallis test). **J-K:** Jumps during stimulation normalized by each flies' baseline. Vertical dashed lines indicate looming events. Single flies are shown in yellow, groups of 3 in orange and groups of 5 in brown. Asterisks indicate significant pairwise differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$). NS indicates nonsignificant differences. Sample sizes $\subset [96, 102]$.

A stronger freezing group effect is observed in FMRF mutants

Figure 8 depicts the behaviors of FMRF mutant flies. These flies exhibited intense running and jump responses to looming in all group conditions. This contrasted with a sharp reduction in freezing limited to flies tested in groups (illustrated in fig. 8A). Single flies froze more than those in groups (fig. 8B-E; $p < 0.001$ for both sexes, Dunn test), whereas no differences were observed between the latter ($p \geq 0.25$ for both sexes, Dunn test). The same pattern was observed for the final proportion of flies freezing (see annex E). As with wild-type flies, we did not find evidence that the group reduction in time spent freezing was due to collisions (see annex F).

Females in all group conditions and individual males increased their walking speed from stimulation to baseline (fig. 8F, H; $p \leq 0.02$, Wilcoxon test), whereas the male groups showed a nonsignificant trend in the same direction ($p \geq 0.15$, Wilcoxon test). In all conditions, average walking speed was significantly higher immediately after looming (fig 9G, I; $p \leq 0.003$, Wilcoxon test). As with wild-type flies, female flies tested alone exhibited a larger increase in this measure, with a significant difference emerging between these and flies in groups of 5 ($p = 0.027$, Dunn test). Males revealed a similar nonsignificant trend ($p = 0.06$, Kruskal-Wallis test).

FMRF mutants exhibited an unusually high number of jumps during both baseline and stimulation, with up to 40% of the flies jumping during looming bins (fig. 8A). Single females tended to jump less than those in groups, but the reverse happened in males (fig. 8J-K). We could also observe a reduction in jumps among female and male single flies throughout the stimulation period that was not observed in groups (fig. 8A). It is unclear whether these differences could explain the main group effect over time spent freezing or if they are a consequence of it.



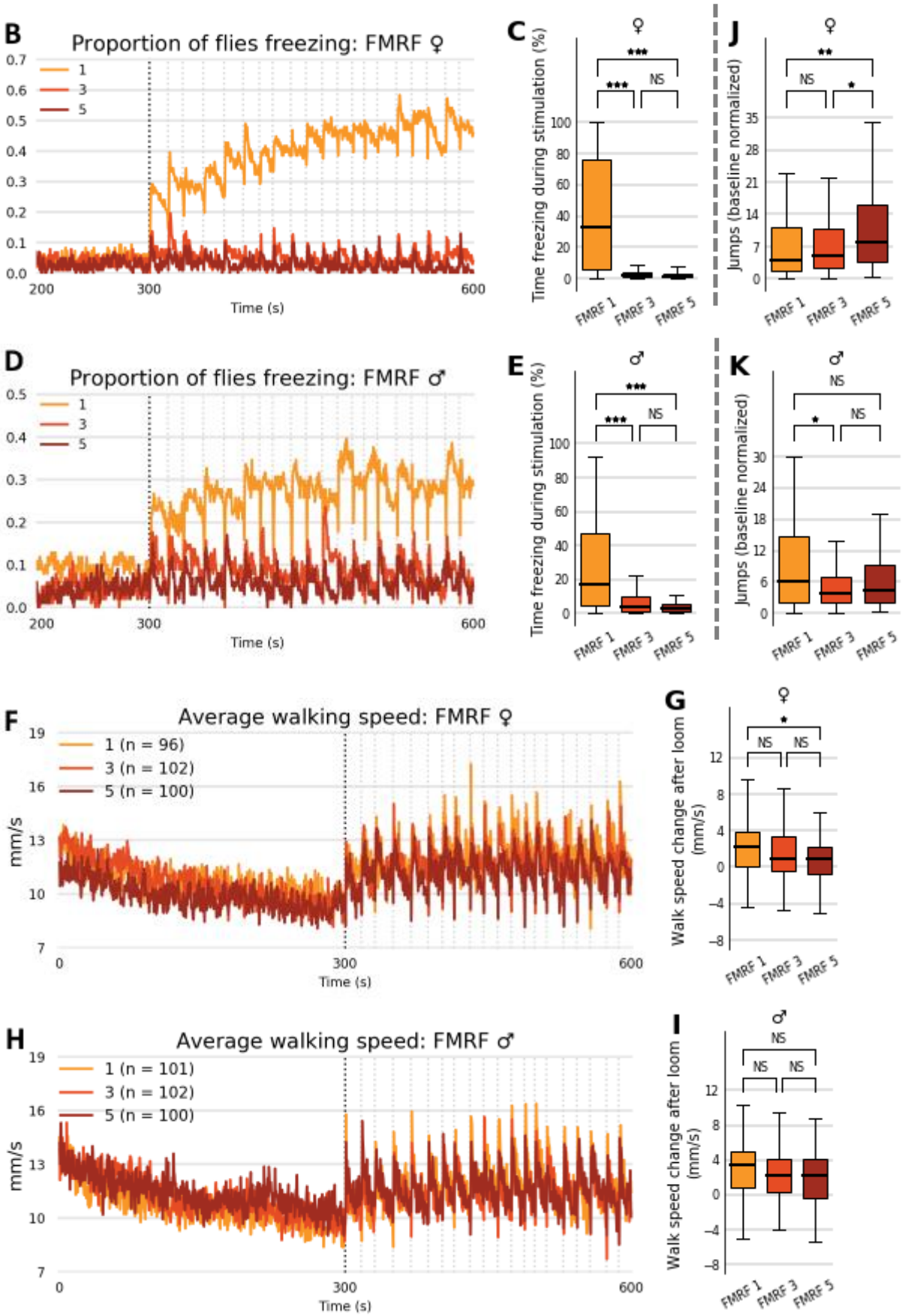

B
Proportion of flies freezing: FMRF ♀
1
3
5
Time (s)
C
♀
Time freezing during stimulation (%)
***
***
NS
FMRF 1
FMRF 3
FMRF 5
J
♀
Jumps (baseline normalized)
**
NS
*
D
Proportion of flies freezing: FMRF ♂
E
♂
K
♂
F
Average walking speed: FMRF ♀
1 (n = 96)
3 (n = 102)
5 (n = 100)
mm/s
G
Walk speed change after loom (mm/s)
H
Average walking speed: FMRF ♂
1 (n = 101)
3 (n = 102)
5 (n = 100)
I

Figure 8. FMRF mutants. **A:** Cumulative proportion of flies freezing (red), grooming (pink), jumping (black) or walking (grey) across the experiment. **B, D:** Proportion of flies freezing across the experiment. **C, E:** Percentage of time spent freezing during stimulation. **F, H:** Average walking speed across the experiment. **G, I:** Average change in walking speed around looming (1s after – 1s before; dashed median indicates nonsignificant difference from zero, $p > 0.05$, Kruskal-Wallis test). **J-K:** Jumps during stimulation normalized by each flies' baseline. Vertical dashed lines indicate looming events. Single flies are shown in yellow, groups of 3 in orange and groups of 5 in brown. Asterisks indicate significant pairwise differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$). NS indicates nonsignificant differences. Sample sizes $\subset [96, 102]$.

Control comparisons

Genetic background strain

We tested the genetic background (y^1w^{67c33}) of Amontillado and FMRF mutants to rule out the possibility of it explaining the effects on freezing observed in either of the lines. The results revealed seemingly normal patterns of freezing among females (fig. 9A), although the time spent freezing was not significantly different between conditions (fig. 9B; $p = 0.06$, Kruskal-Wallis test), probably due to the sample size used (~50). Males, on the other hand, showed less distinct patterns of freezing between group conditions (fig. 9B-C, F; $p = 0.16$, Kruskal-Wallis test), mostly due to a reduction in freezing among flies tested alone. This could explain the shift in freezing observed also in the FMRF and Amontillado individual males (although this was not the case with CCAP flies, who share the same background, Annex D). The fraction of flies freezing by the end of the experiment differed significantly between flies tested alone and in groups of 5 in both sexes, whereas the other conditions were not significantly different (respectively, $p \leq 0.01$ and $p \geq 0.17$, Chi-square test).

As with Amontillado mutants, there was a constant reduction in walking speed throughout the experiment, with the stimulation average being significantly lower than the baseline in all cases (fig. 9E, F; $p \leq 0.007$, Wilcoxon test). Flies did not consistently alter their walking speed around looming either (mdn changes = [-0.12, 0.29], $p \geq 0.37$, Wilcoxon test). Finally, they exhibited few jumps (fig. 9G-H) that were not tightly coupled with looming events (only 55% of the stimulation jumps happened in the 5 seconds after a looming).

These results argue against the role of genetic background effects on the freezing phenotypes observed in both FMRF and Amontillado mutants, at least for females. Due to the 'anomalies' of the male background line, we limited subsequent analysis and experiments to females only.

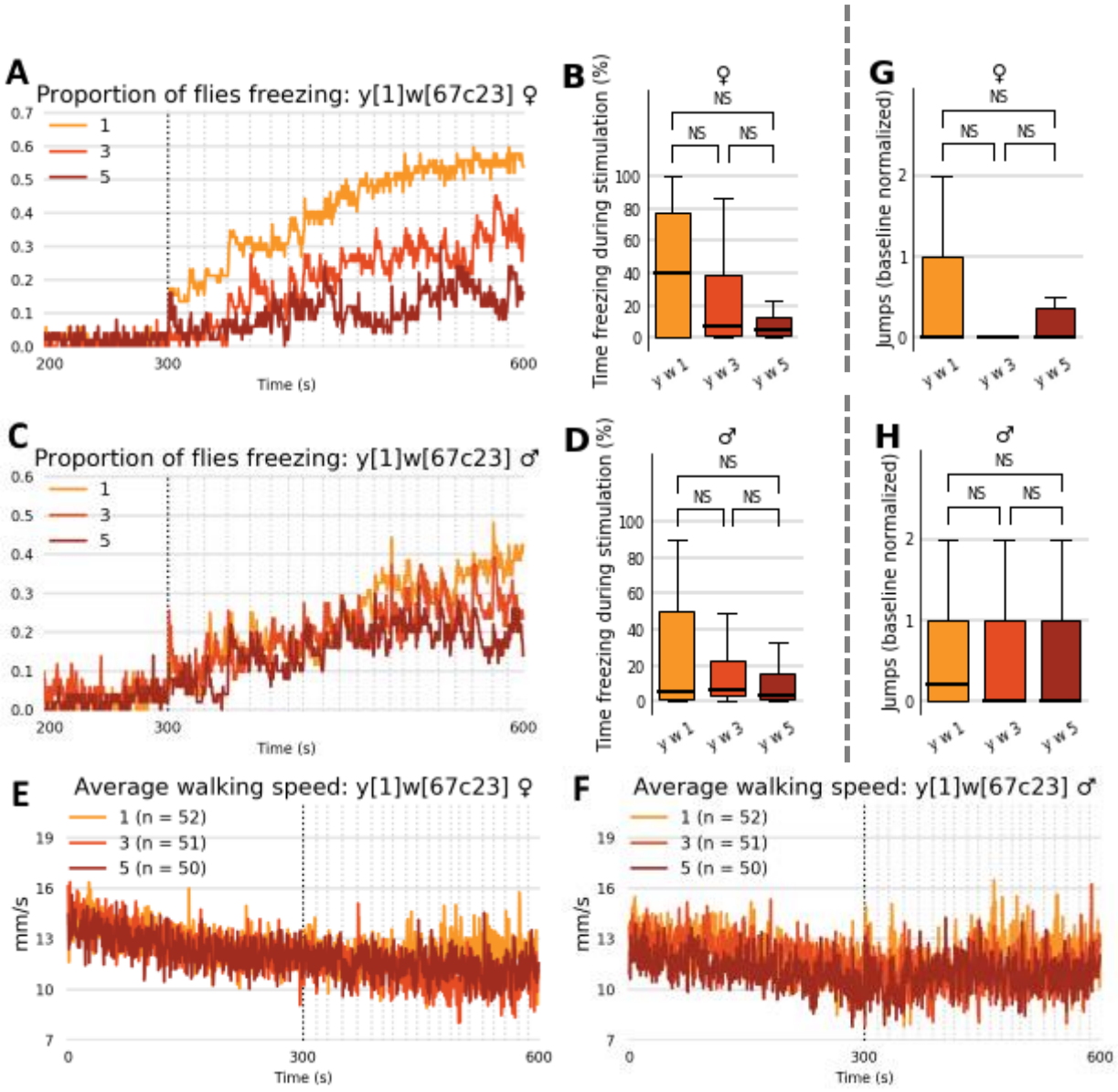


Figure 9. Genetic background strain. **A, C**: Proportion of flies freezing across the experiment. **B, D:** Percentage of time spent freezing during stimulation. **E-F**: Average walking speed across the experiment. **G-H:** Jumps during stimulation normalized by each flies' baseline. Vertical dashed lines indicate looming events. Single flies are shown in yellow, groups of 3 in orange and groups of 5 in brown. Asterisks indicate significant pairwise differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$). NS indicates nonsignificant differences. Sample sizes $\subset$ [50, 52].

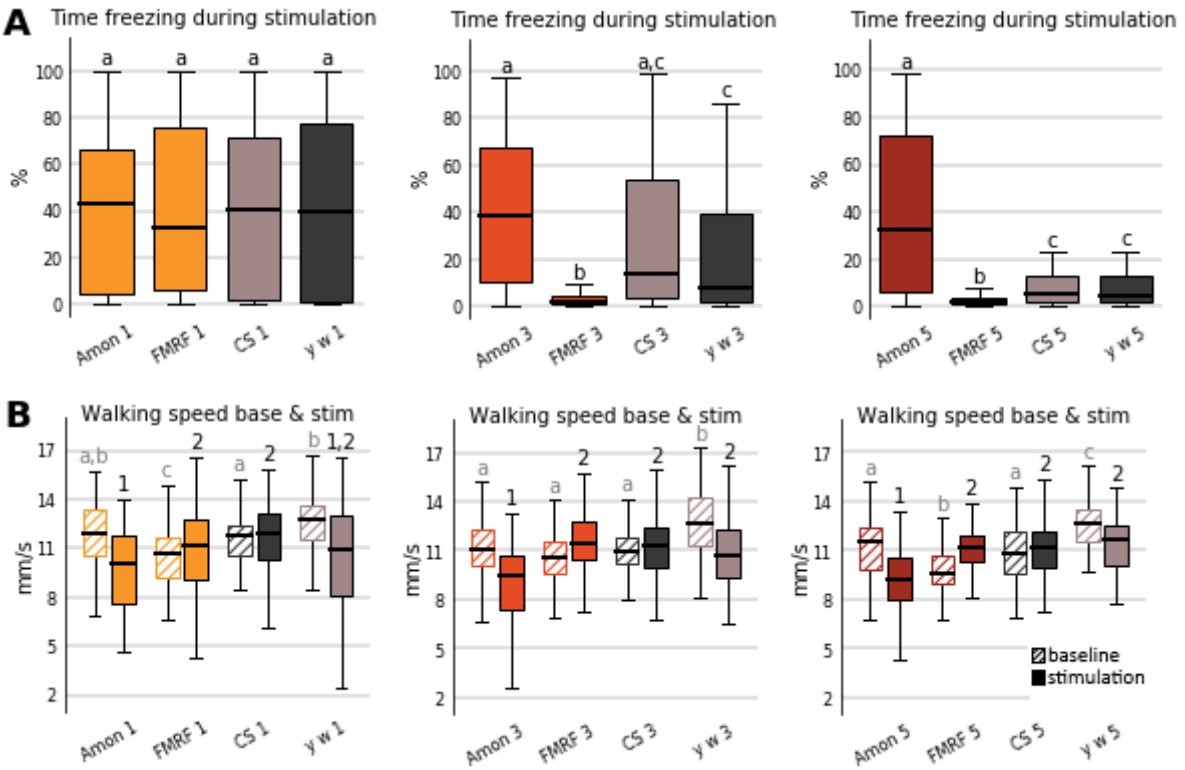
Multiple comparisons

Figure 10 shows the multiple comparisons between the two female controls (wild-type flies and genetic background) and the FMRF and Amontillado mutant strains. Individual FMRF and Amontillado mutants did not differ from individual controls in the time spent freezing (fig. 10A; $p = 0.94$, Kruskal-Wallis test), whereas Amontillado in groups of 5 froze more than the control counterparts ($p < 0.001$, Dunn test) and FMRF in groups of 3 and 5 froze less ($p < 0.001$,

Dunn test), in line with these mutations having group specific effects. A similar trend emerged when comparing the proportion of flies freezing by the end of the experiment (Annex E).

FMRF mutants generally exhibited a low average walking speed during baseline, but upon stimulation their speed equaled that of control flies (fig. 10B). In contrast, Amontillado mutants showed a reduction in walking speed from baseline to stimulation, with the latter being lower than both controls in groups of 3 and 5 (fig. 10B, $p < 0.001$, Dunn test). This pattern was also observed in the genetic background flies, with the difference that their average speed during baseline was generally higher than other strains. The change in speed around looming followed similar albeit less distinct patterns (fig. 10C).

The number of jumps initiated by controls during stimulation paled in comparison to that of FMRF mutants, even after controlling for baseline levels (fig. 10D; $p < 0.001$, Dunn test). Despite jumping less, wild-type flies showed a larger shift of jumps towards the stimulation period (fig. 10E; $p < 0.001$, Dunn test), suggesting that their response is more specific to looming. In contrast, Amontillado mutants and the background line showed weaker responses, jumping less (fig.10D), and having their jumps more evenly distributed across baseline and stimulation (fig. 10E).



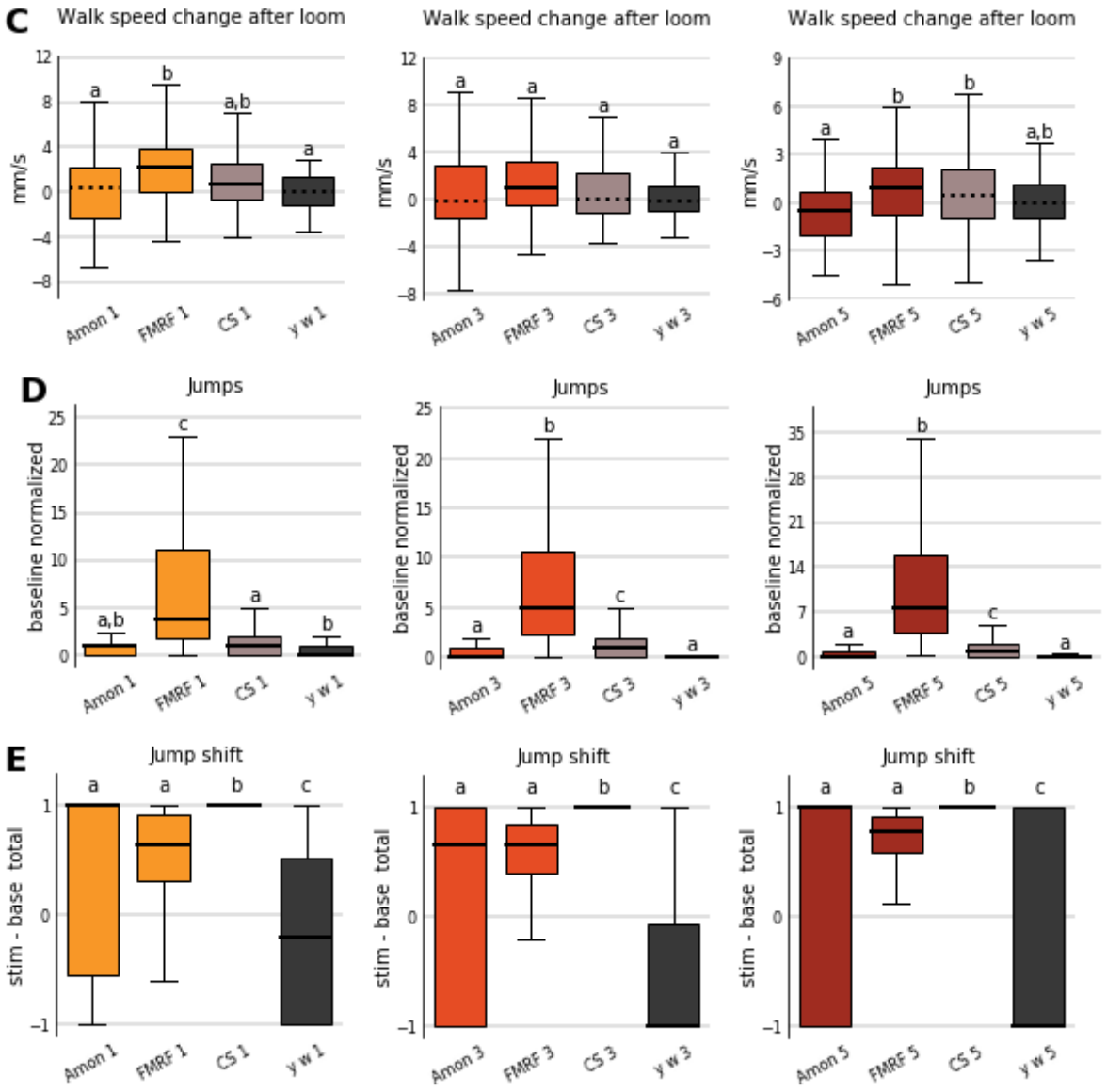


Figure 10. Comparison between controls and Amontillado and FMRF mutants. **A:** Percentage of time spent freezing during stimulation. **B:** Average walking speed during baseline (dashed) and stimulation (solid). **C:** Average change in walking speed around looming (1s after – 1s before; dashed median indicates nonsignificant difference from zero, $p > 0.05$, Kruskal-Wallis test). **D:** Jumps during stimulation normalized by each flies' baseline. **E:** Jump shift [(jumps in stimulation - jumps in baseline) / total]. Single flies are shown in yellow, groups of 3 in orange and groups of 5 in brown. Different letters or numbers indicate significant pairwise differences ($p \leq 0.05$). P-values were corrected by a factor of 4 (2 mutants x 2 controls). Sample sizes $\subset [88, 102]$ except for the genetic background strain where sample size $\subset [50, 52]$.

These results confirm that the strength of the group effect on freezing in the Amontillado and FMRF mutants is indeed distinct from that of wild-type and genetic background strains. Two potential explaining factors emerged from this analysis: a slower walking speed during stimulation in amontillado mutants, and strong jump and running responses in FMRF flies. The potential implications of these observations will be elaborated in the discussion. The following sections

describe a set of parallel experiments designed to confirm the FMRF hit, using a deficiency line and RNA interference.

FMRF Deficiency and RNA interference

FMRF deficiency line retains the mutant phenotype

To confirm the role of the FMRF gene in the freezing phenotype of groups, we crossed our mutants with a deficiency line where the region of DNA that contains this gene is deleted. We successfully replicated the results obtained with FMRF mutants. Flies in groups of 3 and 5 showed lower levels of freezing (fig. 11B-C) and spent less time freezing than either of the parental controls (fig. 11E-F; $p \leq 0.011$, Dunn test), whereas flies tested alone showed a robust freezing behavior that was similar to that of parental controls (fig. 11A, D; $p = 0.20$, Kruskal-Wallis test). A similar trend was observed when comparing the proportion of flies freezing by the end of the experiment, although only the groups of 3 remained statistically different from controls following correction for multiple comparisons (see Annex E).

Flies tested in groups showed a large increment from the baseline walking speed (which was similar to that of +/Def control) to stimulation (which was similar to that of FMRF/+ control). In contrast to groups, FMRF/Def flies tested alone exhibited a reduction of walking speed during the stimulation period (fig 11G-I). In all three lines and group conditions, flies increased their walking speed around looming, the magnitude of which was generally higher in FMRF/+ flies and similar in FMRF/Def and +/Def flies (data not shown).

Comparably to the FMRF mutants, the FMRF/Def flies exhibited high levels of jumping throughout the entire experiment, which were not observed in the parental lines ($p < 0.001$, Dunn test). After adjusting for baseline, the levels of jumping in stimulation became similar to those of the +/Def control, and generally lower than the FMRF/+ control, suggesting that the high levels of jumps are not entirely due to looming exposure (fig 11J-L).

Taken together, these results corroborate that the group effect on freezing observed in our mutant line is indeed related to the activity of the FMRF gene region. Similarly, this data supports the findings that FMRF knockdown increases the propensity to jump regardless of looming exposure, and possibly the magnitude of change in average walking speed from baseline to stimulation.

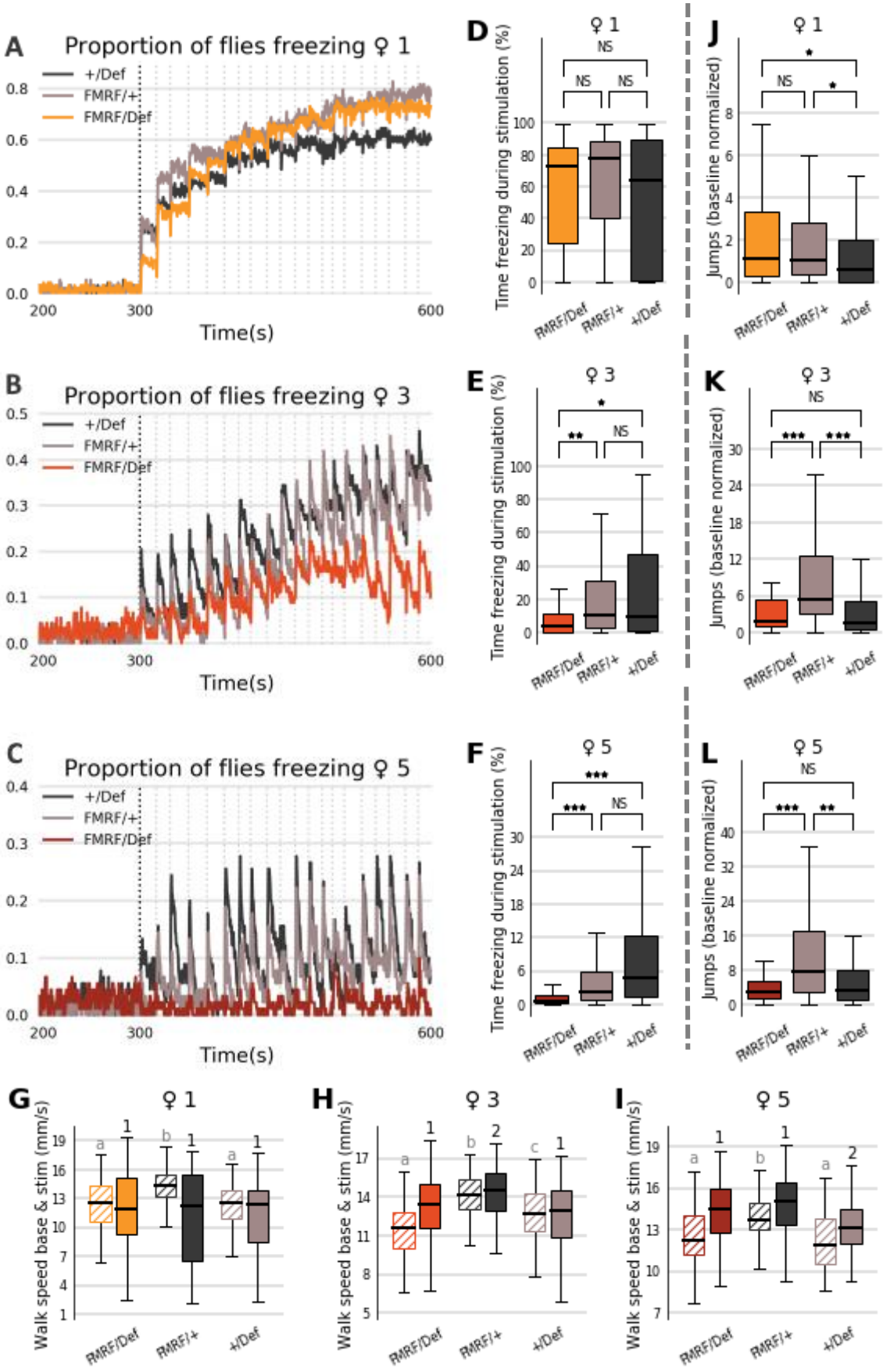
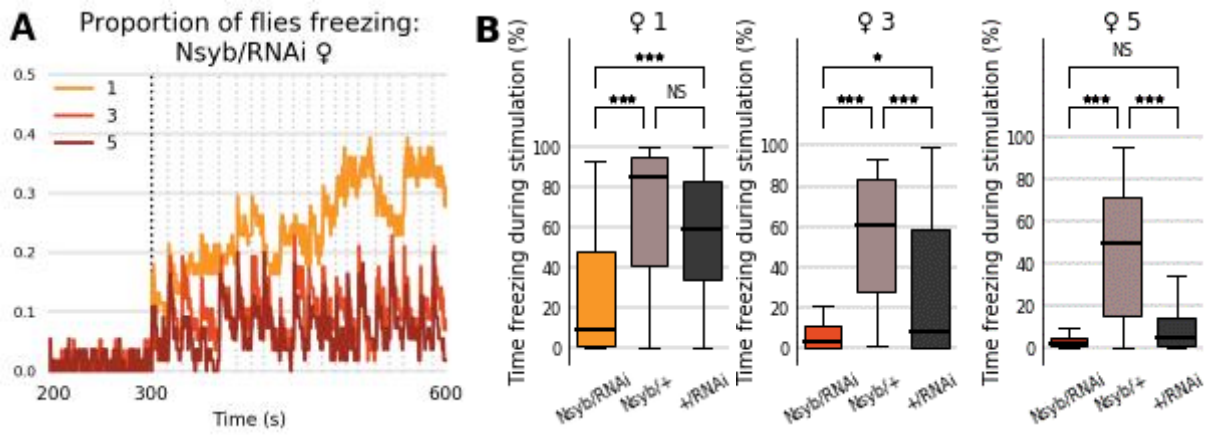

A
Proportion of flies freezing ♀ 1
+/Def
FMRF/+
FMRF/Def
Time(s)
B
Proportion of flies freezing ♀ 3
C
Proportion of flies freezing ♀ 5
D
♀ 1
Time freezing during stimulation (%)
E
♀ 3
F
♀ 5
J
Jumps (baseline normalized)
K
L
G
Walk speed base & stim (mm/s)
H
I
RMRF/Def
RMRF/+
+/Def

Figure 11. FMRF Deficiency strain. **A-C:** Proportion of flies freezing across the experiment, from 200 s onwards (cropped baseline). Vertical dashed lines indicate looming events. **D-F:** Percentage of time spent freezing during stimulation. **G-I:** Average walking speed in baseline (dashed) and stimulation (solid). **J-L:** Jumps normalized by each flies' baseline. Single flies (A, D, G, J), groups of 3 (B, E, H, K), groups of 5 (C, F, I, L). Asterisks indicate significant pairwise differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$). NS indicates nonsignificant differences. Different letters or numbers indicate significant pairwise differences (< 0.05). Sample sizes ⊂ [90, 93].

FMRF RNA interference failed to reproduce the mutant phenotype

To further confirm the role of FMRF on the social modulation of defensive responses, and also to determine whether this effect is neuronally related, we attempted to knock down FMRF using a pan-neuronal Nsyb-Gal4 line to drive a RNA interference (RNAi) UAS line. The main results for this experiment are depicted in figure 12.

Flies tested alone froze considerably little compared to the FMRF mutant and FMRF deficiency lines, despite still spending more time freezing than flies tested in groups (fig. 12A, $p \leq 0.048$, Dunn test). The time spent freezing among flies tested alone was also lower than both parental controls (fig.12 B; $p < 0.001$, Dunn test). The same results were obtained when comparing the proportion of flies freezing by the end of the experiment (Annex E). In addition, individual flies displayed a robust increase in walking speed after looming that was not present in those tested in groups (fig.12 C; $p < 0.001$, Dunn test) or in controls tested alone (fig. 12D; $p \leq 0.026$, Dunn test), which could suggest a shift from freezing to running that is specific to the individual flies' condition and which is a result of the genetic manipulation. Finally, these flies jumped little in comparison to controls and the FMRF and deficiency lines (data not shown).



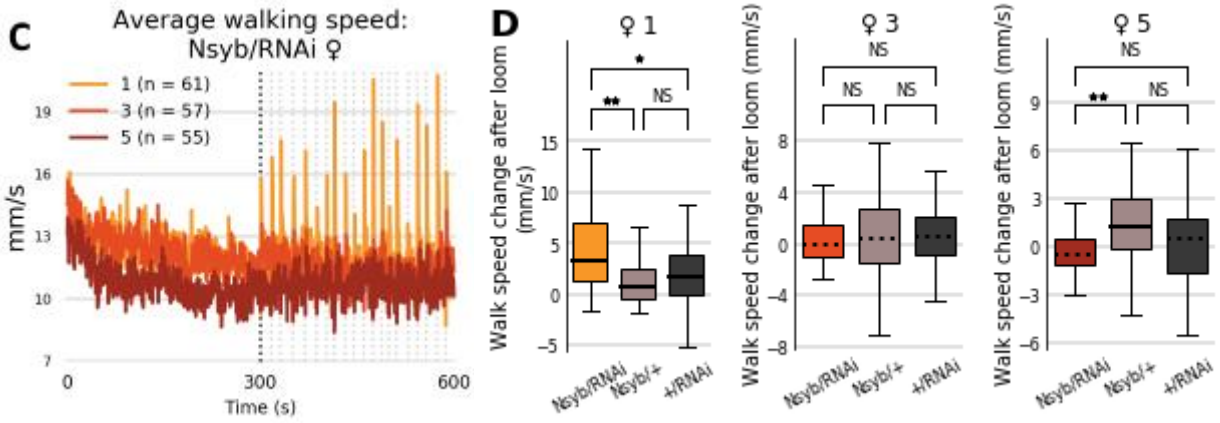


Figure 12. Pan-neuronal knockdown of FMRF using Nsyb Gal4 x FMRF RNAi UAS. **A:** Proportion of flies freezing across the experiment. **B:** Time spent freezing during stimulation. **C:** Average walking speed across the experiment. **D:** Average change in walking speed around looming (1s after – 1s before; dashed median indicates nonsignificant difference from zero, $p > 0.05$, Kruskal-Wallis test). Vertical dashed lines indicate looming events. Single flies are shown in yellow, groups of 3 in orange and groups of 5 in brown. Control flies are shown in grey and black, irrespective of group condition. Asterisks indicate significant pairwise differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$). NS indicates nonsignificant differences. Different letters or numbers indicate significant pairwise differences (< 0.05). Sample sizes $\subset [55, 61]$.

Because the Nsyb driver already exerted a strong effect on the defensive behavior of flies (i.e., a strong increase in freezing especially notable among flies in groups, fig.12 B), we were not able to draw any strong conclusions regarding the phenotype of the Nsyb/RNAi flies. To overcome this issue, we tried using a different neuronal driver line — elav — with the same RNAi construct.

Figure 13 depicts the main results from this second experiment. Contrary to the Nsyb driver, we did not notice any issue with the elav parental line. Elav/RNAi flies showed a main group effect on time spent freezing, although we could not distinguish between specific group conditions after correcting for multiple comparisons (fig. 13A; $p = 0.031$, Kruskal-Wallis test; $p \geq 0.05$, Dunn test). The time spent freezing did not differ between that of parental controls in any group condition either (fig. 13B; $p \geq 0.15$, Kruskal-Wallis test). Similar results were obtained when comparing the proportion of flies freezing by the end of the experiment (Annex E). Once again, we could observe transient peaks in walking speed after looming that were specific to the alone condition (fig. 13C), but these were less common than in the Nsyb/RNAi line and were not statistically different from the group conditions after correcting for multiple comparisons ($p = 0.036$, Kruskal-Wallis test; $p \geq 0.06$, Dunn test), nor were they different from the parental controls tested alone (fig. 13D, $p = 0.30$, Kruskal-Wallis test). These flies also displayed little jumping (data not shown). The lack of information about the efficacy of the two lines used renders the interpretation of these results difficult.

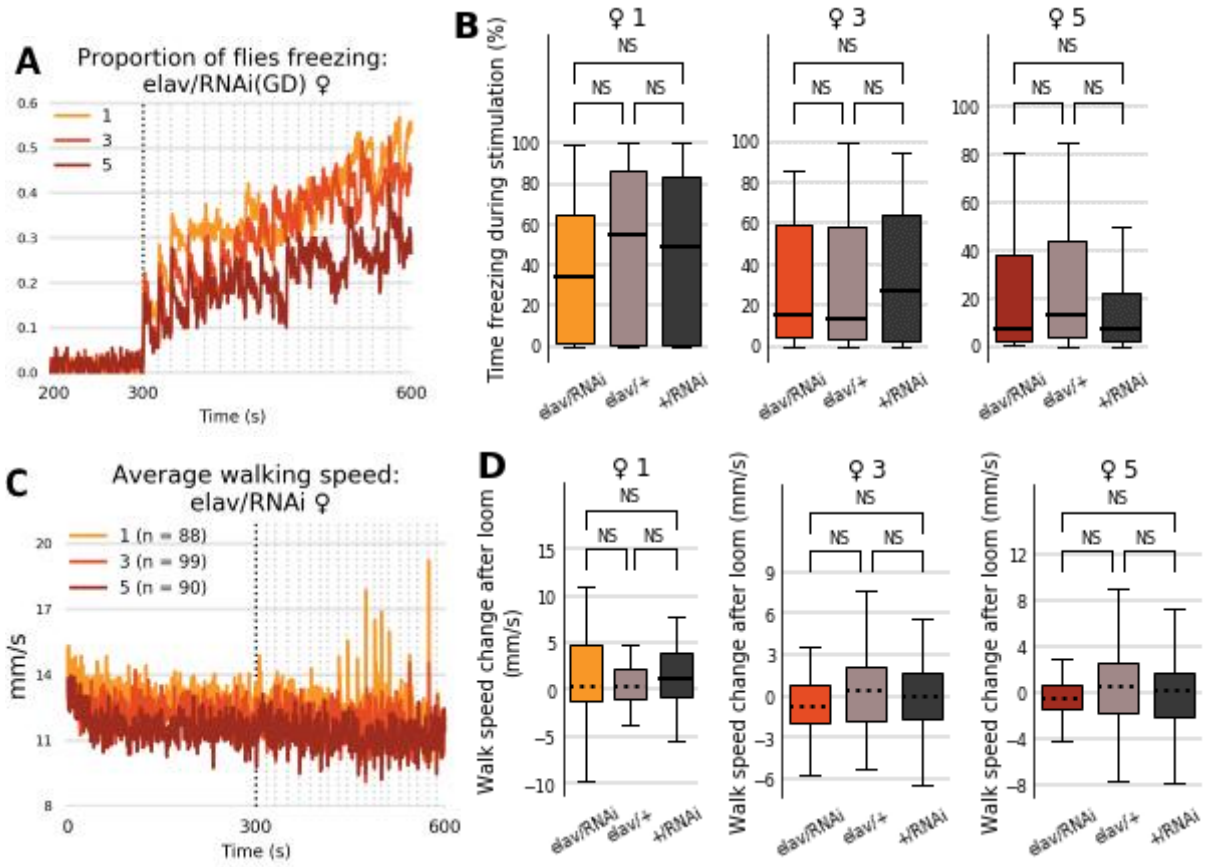


Figure 13. Pan-neuronal knockdown of FMRF using elav Gal4 x FMRF RNAi UAS. **A:** Proportion of flies freezing across the experiment. **B:** Time spent freezing during stimulation. **C:** Average walking speed across the experiment. **D:** Average change in walking speed around looming (1s after – 1s before; dashed median indicates nonsignificant difference from zero, $p > 0.05$, Kruskal-Wallis test). Vertical dashed lines indicate looming events. Single flies are shown in yellow, groups of 3 in orange and groups of 5 in brown. Control flies are shown in grey and black, irrespective of group condition. Asterisks indicate significant pairwise differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$). NS indicates nonsignificant differences. Different letters or numbers indicate significant pairwise differences (< 0.05). Sample sizes ⊂ [88, 90].

FMRF mutants are more sensitive to changes in looming intensity

Our experiments suggested that FMRF mutant flies' weaker freezing phenotype was limited to group conditions, a hypothesis that was further supported with the results from the FMRF deficiency strain. However, it could be the case that single flies also have a tendency towards less freezing but that our protocol was not robust enough to capture it. To test this hypothesis, we ran a new set of experiments with single FMRF and wild-type flies in which we varied looming intensity (by changing the stimulus color to be 27%, 9% and 3% black). If the FMRF mutation also has an effect on individuals' tendency to freeze, we would expect their responses to diverge from wild-type flies as looming becomes fainter.

In line with our hypothesis, FMRF flies exhibited considerably less freezing in the 27% and 9% looming conditions compared to wild-type flies. After correction for multiple comparisons, the time spent freezing was significantly different in the 27% condition but not in the 9% (fig. 14A; respectively $p = 0.041$ and $p = 0.14$, Kruskal-Wallis test), while the proportion of flies freezing by the end was different in both conditions (fig. 14B, $p \leq 0.0014$, Chi-square test). Surprisingly, when comparing these results with those from the previous experiments (100% black), the largest change belonged to wild-type flies who were now freezing substantially more. We believe that these differences are most likely due to inter-experimental factors.

The freezing behavior displayed by the two strains converged in the 3% looming condition, which may be close to the flies' perceptual threshold. Because the time spent freezing in the 9% condition was significantly higher than in the 3% in both strains ($p \leq 0.027$, Kruskal-Wallis test), we are confident that flies were still perceiving the 9% black stimuli.

We also observed an interaction between strain and looming condition regarding changes in walking speed, although they never attained statistical significance (fig. 14C-D). Jumps and change in time spent grooming gradually converged to zero, which suggests that other defensive responses besides freezing were also being attenuated (fig. 14E-F).

These results provide preliminary support for the hypothesis that the FMRF mutation confers a higher threshold for freezing and/or decreases the salience of the looming stimuli. It is plausible that the group manipulations tap into this aspect, leading to the floor levels of freezing exhibited by FMRF mutants (which are still below those detected here). Such conclusions are, however, strongly limited by the lack of a simultaneous control condition (100% black) and the intermediate sample size used (~ 45).

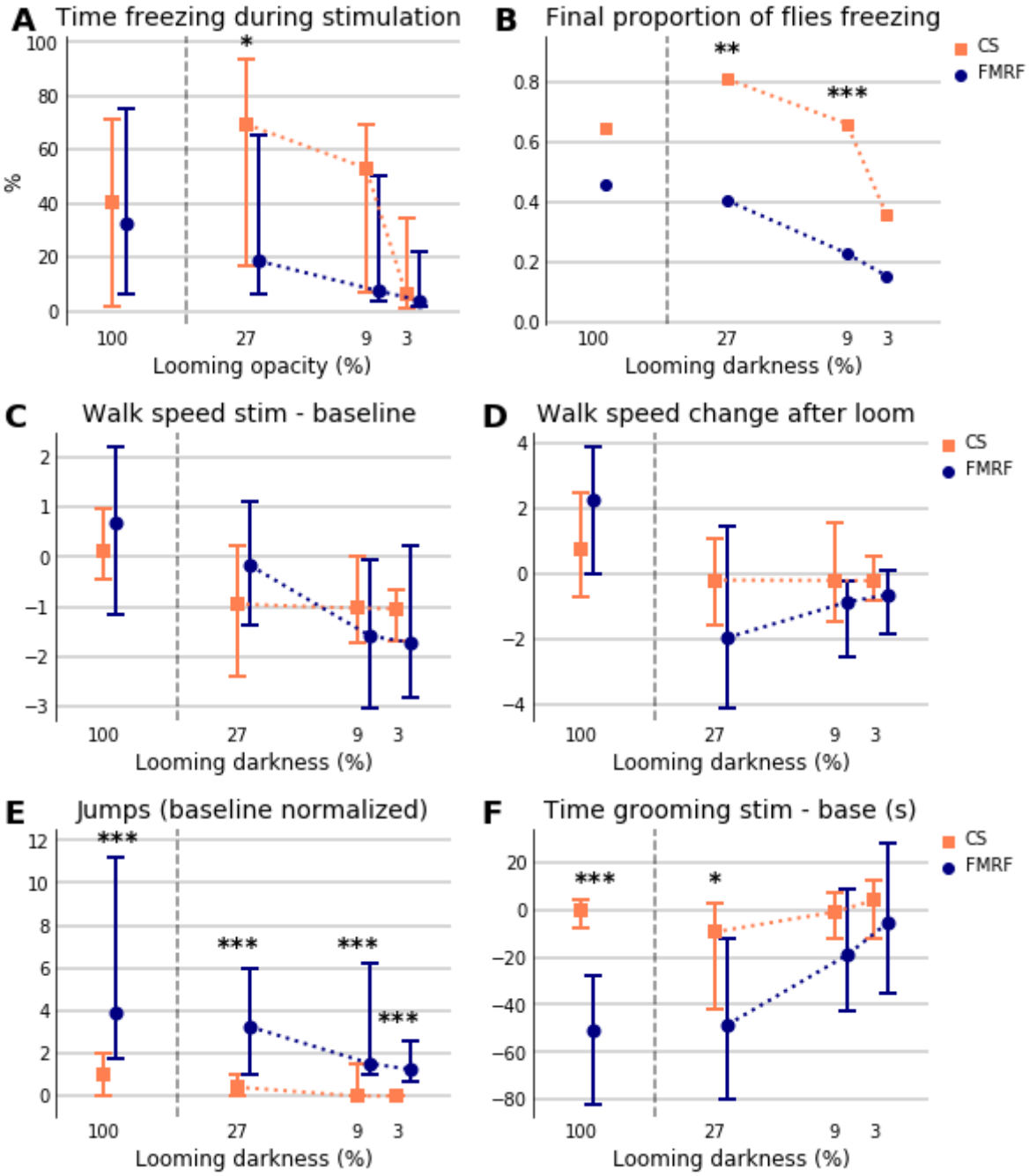


Figure 14. Wild-type flies and FMRF mutants under conditions of varying looming intensity. **A:** Percentage of time spent freezing during stimulation **B:** Proportion of flies freezing by the end of the experiment. **C:** Change in average walking speed from baseline to stimulation. **D:** Average change in walking speed around looming (1s after – 1s before). **E:** Number of jumps during stimulation, normalized by each flies' baseline. **F:** Time spent grooming during stimulation. Data from wild-type flies is shown in orange and data from FMRF mutants in blue. Except for plot B, the central data points refer to median values and the whiskers extend between the 25th and 75th percentiles. Asterisks indicate significant pairwise differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$). Sample sizes $\subset [42, 46]$ except for 100% black looming condition where sample size = 88.

Discussion

The present work aimed to assess the potential role of neuropeptide signaling on the social modulation of defensive responses to inescapable looming stimuli in *Drosophila*, through an informed screen of neuropeptide and neuropeptide processing mutants.

In a first stage, we successfully replicated previous findings from our group, showing that flies are more prone to break from freezing as the number of conspecifics in the same arena increases, leading to lower total time spent freezing and lower probability of freezing by the end of the experiment. This reduction was not accompanied by an increase in other defensive responses (walking velocity or jump frequency) suggesting a buffering effect by the social context.

Three of the mutant lines tested were unresponsive to looming. We speculate that two cases were due to an interaction between the Minos insertion and the genetic background used to create most of the mutant lines available; this interpretation was substantiated by other experiments not reported here, which led us to discontinue the plans for a more extensive genetic screen. Of the responsive lines tested, we focused on Amontillado and FMRF, who displayed altered responses in groups. We further limited our analysis to females due to potential issues with the male genetic background.

Amontillado enzyme

We observed a suppression of the group effect on freezing in Amontillado mutants. Flies in groups of 3 and 5 displayed sustained levels of freezing similar to those of single Amontillado and control flies. We did not observe any group differences in other behaviors. However, flies in all conditions exhibited a weak modulation of walking velocity to looming, with non-freezers walking considerably slowly by the end of the experiment. The potential implications of this observation are further considered below.

Amontillado is a homologue to the mammalian Protein Convertase 2 (PC2). It plays a crucial role in the early synthesis of many neuropeptides, being responsible for the functional cleavage of proproteins at predetermined sites. Based on the limited role of homologous enzymes in the fly genome, Wegener and colleagues (2011) argue that Amontillado is the main proprotein

convertase in the fly. As evidence of this, Amontillado is coexpressed with neuropeptides in the CNS and gut endocrines, and immunostaining and mass spectroscopy experiments with mutants show a reduction in all the quantified neuropeptides (Rhea et al., 2010, Wegener et al., 2011). It is therefore plausible that the phenotype observed is caused by the knockdown of downstream products and not specific to the Amontillado mutation.

FMRF neuropeptides

Among such downstream products of Amontillado are the FMRF neuropeptides. A mutant line for the precursor of these peptides revealed a phenotype in our screen, albeit in the opposite direction. FMRF mutant flies tested in groups displayed considerably lower levels of freezing than the respective wild-type flies in the same social conditions, whereas flies tested alone seemed to be unaffected. Also in contrast with the Amontillado mutants, FMRF flies showed an increase in walking velocity and jump responses to looming.

We successfully replicated this phenotype after crossing the mutant with a deficiency line where the FMRF gene region has been deleted. When these flies were tested in groups they showed a significant reduction in sustained freezing compared to the parental controls, whereas single flies did not. In contrast, we failed to replicate the FMRF freezing phenotype by using RNAi with two neuronal Gal4 drivers. Nsyb/RNAi flies tested alone showed a reduction in freezing, although this response was still larger than in flies tested in groups. It is possible that the RNAi promoted a reduction in freezing among all conditions, but that due to a floor effect the group differences were effectively narrowed. However, because the Nsyb parental control exhibited a phenotype of its own, it is not possible to assess whether these effects were due simply to the FMRF knockdown or emerged from an undesired interaction between this and the effects of the Nsyb driver. We did not notice any abnormalities with the parental lines when combining the elav driver with the same RNAi construct, but the elav/RNAi flies still did not replicate the FMRF phenotype (we observed a weaker group effect, but no differences from parental controls). Because we do not know the extent to which these lines disrupted FMRF function, we consider this data to be inconclusive and not necessarily contrary to that obtained from the mutant and deficiency lines. To overcome these limitations, it may be necessary to employ other driver and/or RNAi lines, as well as to measure the extent to which these alter the concentration of FMRF peptides.

Despite having been the first insect neuropeptide gene to be cloned, the functions of FMRF in *Drosophila* are still not completely understood (see Nässel & Winther, 2010). It encodes for a prepropeptide that originates eight peptides that are present in the neurosecretory cells of the fly, and which seem to be functionally redundant (Hewes et al., 1998, but see Cazzamali & Grimmelikhuijzen., 2002). Functional studies have shown FMRF to modulate muscle contractions (Hewes et al., 1998; Kaminski et al., 2002; Clark et al, 2008), and to be involved in locomotor defensive responses to intense light in larvae (Klose et al.,2010) and air puffs in adults (Kiss et al., 2013), as well as heat stress induced sleep in adults (Lenz et al., 2015). In mammals, FMRF peptides act on G-protein coupled receptors (GPCRs), regulating muscle contraction, nociception, feeding, learning and memory (Panula et al., 1996). In addition, the fly FMRF receptor might also be related to mammalian thyroid hormone receptor (Cazzamali & Grimmelikhuijzen, 2002; Meeusen et al., 2002).

Underlying mechanisms

Given that the Amontillado and FMRF molecules share the same biological pathway, it is perhaps surprising that their respective mutants would display opposite phenotypes. One explanation for this is that other neuropeptides downstream of Amontillado exert actions that counteract and override the effects of FMRF knockdown. We found some tentative evidence in this direction from another mutant strain. CCAP male mutants tested in groups of 5 showed a trend towards higher levels of freezing than those in groups of 3 (annex D).

Alternatively, the effects observed in one or both mutant strains could have emerged not through direct changes in how the individuals respond to the presence of others, but indirectly through the changes in the group behavior of the flies. Since the Amontillado mutant flies tended to slow down or engage in sustained freeze, while FMRF mutants increased their walking speed and jump probability towards looming, flies from each line ended up experiencing drastically different social contexts. It is possible that these contexts are sufficient to drive the specific freezing phenotypes observed among flies tested in groups. If this is indeed the case, we would expect them to vanish when single mutant flies are placed in a group of wild-type flies or, conversely, to emerge among wild-type flies placed in a group of Amontillado or FMRF mutants.

Yet another important consideration is that despite having captured these effects using a social manipulation, they need not reflect a social phenomenon. Indeed, preliminary findings described in this work suggest that the FMRF mutation has a subtle disrupting effect on the freezing behavior of flies tested alone, which becomes more apparent in conditions where the threats are less intense. It is plausible that the group manipulations act on top of this individual phenotype, leading to the floor levels of freezing observed among FMRF mutants tested in groups of 3 and 5.

Any of these scenarios could offer important clues to understand the broader question of how threat perception, decision making and defensive response are shaped and implemented in *Drosophila*. Freezing behavior requires a perceptual threat detection mechanism that is both robust and specific, as to overcome the high costs of predation without impeding basic exploratory and social behaviors. The adequate choice of freezing over alternative defenses also requires the ability to gauge the availability of escape, the distance of the threat, the time elapsed since the last encounter, possibly while also monitoring internal cues such as the levels of energy. As evidenced throughout this work, the presence of conspecifics could be yet another cue used by the flies, perhaps to decide when is it safe to stop freezing. Finally, the implementation of freezing places challenges of its own, as flies need to withstand their posture in adverse conditions (e.g., while being pushed by other flies, in an unbalanced position, over slippery surfaces). The findings presented in this work suggest that one or more of these processes might involve neuropeptide modulation — and more specifically FMRF signaling. More research is required to determine its exact role and the underlying biological mechanisms.

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Annexes

A. Fly strains

Mutant strains were obtained from the Bloomington Drosophila Stock Center: Mi{ET1}amon^{MB00756}, Mi{ET1}amon^{MB04710}, P{SUPor-P}FMRFa^{KG01300}, P{EPgy2}CCAP^{EY15558}, Mi{ET1}Dh44^{MB07006}, and Mi{MIC}Ms^{MI11411}; as was the deficiency line Df(2R)BSC152/CyO. Control strains included Canton-S and y^{1}w^{67c33}. The FMRF RNAi - UAS lines were obtained from the Vienna Drosophila Resource Center (stocks GD37965 and KK103981). The driver lines elav-Gal4 and Nsyb-Gal4 were provided by the Ribeiro lab.

B. Fly chambers specifications

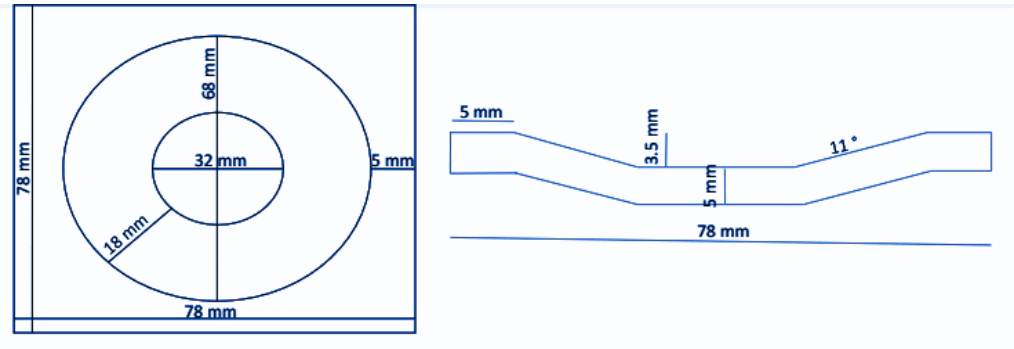

Figure A1. Specifications of the fly chambers. Top view is presented on the left and side view on the right.

C. Validation of the automatic behavior classification

In order to evaluate the accuracy of the automatic classification, 50 unique events were randomly selected for each behavior, sex and experiment point (stimulation and baseline). The following tables show the percentage of true positives, as well as the total number of events identified automatically. In addition to each, there is a description of the most common events deemed false positives. The identification of defensive events (freezing and jumping) during stimulation was highly accurate, while a few false positives creeped during baseline. The opposite happened for the non-defensive behavior of grooming.

	True positive Jump events			
	Baseline	Baseline total events	Stimulation	Stimulation events
Females	40%	54	96%	417
Males	56%	243	94%	1506
False positives	Falling from the ceiling, running, tracker jumping			

	True positive Freezing events			
	Baseline	Baseline events	Stimulation	Stimulation events
Females	20%	795	100%	45594
Males	18%	2276	98%	47861
False positives*	Grooming, quick stop to avoid collision with incoming fly, stop when reaching wall. *For baseline, these are considered periods of immobility, and not false positives, as freezing never occurs during it.			

	True positive Grooming events			
	Baseline	Baseline events	Stimulation	Stimulation events
Females	80%	4794	32%	4195
Males	92%	13358	66%	8809
False positives	Stop walking (baseline), breaking from freezing, another fly crossing ROI while freezing, tracker jittering during freezing			

D. CCAP Mutants

CCAP mutants had a sexually dimorphic phenotype, with female flies exhibiting substantially less freezing than males (fig. A2: A,C). In males, flies tested in groups of 5 showed a trend towards higher freezing than those in groups of 3, although neither the difference in time spent freezing (G3 mdn = 5.8 s, iqr = [2.1; 48.3]; G5 mdn = 15.3 s, iqr = [4.1; 64.5]; p = 0.23, Dunn test), nor the difference in the final proportion of flies freezing (G3 = 0.25, G5 = 0.41, p = 0.33, Chi-square test) reached statistical significance.

Visual inspection of the average walking speed plots also suggested a stronger response by males than females (fig. A2: B,D). In contrast with their weaker freezing phenotype, males in groups of 3 showed the largest increment in average walking speed from baseline to stimulation, with the latter estimate being higher than in flies tested alone and those in groups of 5 (G1: mdn = 10.2 mm/s iqr = [7.93. 12.3]; G3: mdn = 11.74 mm/s, iqr = [10.6; 13.7]; G5: mdn = 10.5 mm/s, iqr = [9.4; 12.0]; $p \leq 0.016$, Dunn test). CCAP flies of both sexes exhibited little jumping in both phases of the experiment, and regardless of group condition (all medians = 0).

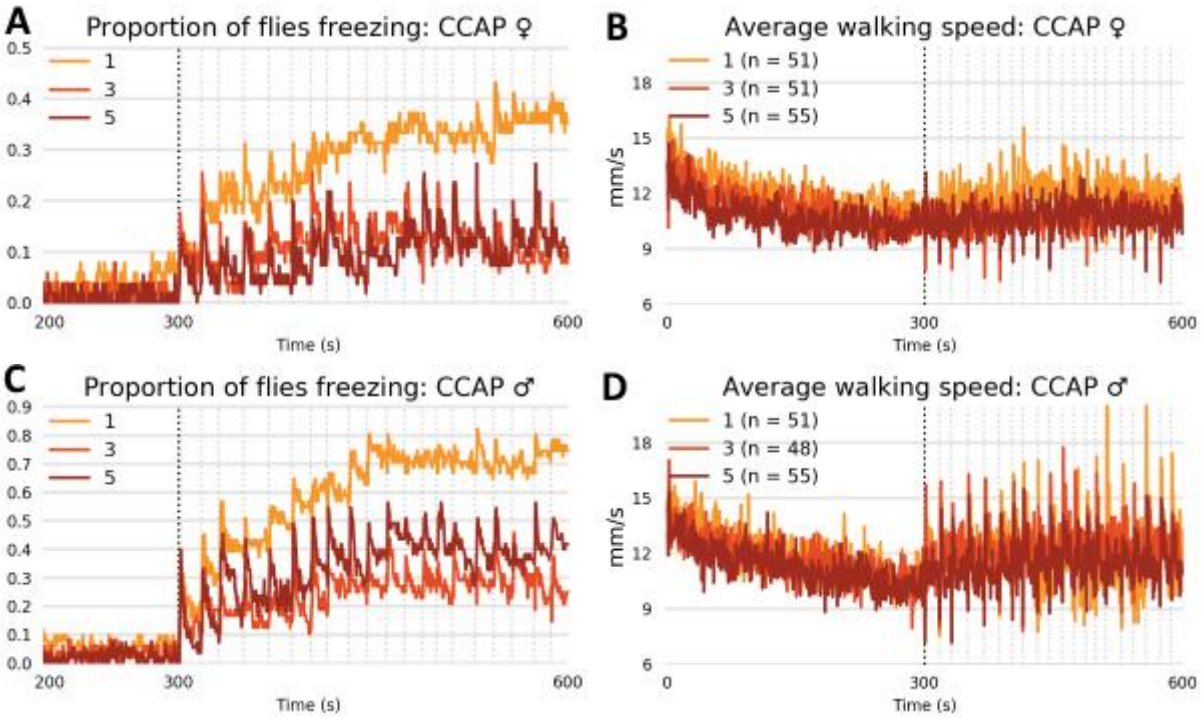

Figure A2. CCAP mutants. **A,C:** Proportion of flies freezing across the experiment. **B,D:** Average walking speed across the experiment. Vertical dashed lines indicate looming events. Females are shown on top and males on bottom. Single flies are shown in yellow, groups of 3 in orange and groups of 5 in brown. Sample sizes $\subset$ [48, 55].

E. Multiple comparisons of proportion of flies freezing by the end of the experiment

Table A1. Proportion of flies freezing by the end of the experiment (within strains)

	Proportion of flies freezing by the end (%)			Chi-square adjusted p-value			
Strain	Alone	Groups 3	Groups 5	All	1 vs 3	1 vs 5	3 vs 5
CS ♀	64.7	43.3	20.0	<0.001	0.020	<0.001	0.004
CS ♂	74.7	26.7	1.1	<0.001	<0.001	<0.001	<0.001
Amontillado ♀	46.5	44.8	45.0	0.96	-	-	-
Amontillado ♂	17.6	12.5	8.0	0.12	-	-	-
FMRF ♀	45.8	3.9	0	<0.001	<0.001	<0.001	0.40
FMRF ♂	28.71	3.9	5.0	<0.001	<0.001	<0.001	1
CCAP ♀	35.3	7.8	9.0	<0.001	0.005	0.007	1
CCAP ♂	74.5	25.0	41.8	< 0.001	<0.001	0.004	0.33
y^1w^{67c33} ♀	53.8	33.3	16.0	<0.001	0.17	<0.001	0.22
y^1w^{67c3}	42.3	23.5	14.9	0.004	0.21	0.010	1
FMRF/Df ♀	73.1	10.0	0	<0.001	<0.001	<0.001	0.019
FMRF/ + ♀	77.4	30.1	7.8	<0.001	<0.001	<0.001	<0.001
+/Def ♀	60.2	35.5	6.7	<0.001	0.003	<0.001	<0.001
Nsyb/RNAi ♀	27.9	7.0	1.8	<0.001	0.020	<0.001	1
Nsyb/+ ♀	79.3	70.0	61.8	0.11	-	-	-
+/RNAi(1) ♀	77.6	41.7	7.3	<0.001	<0.001	<0.001	<0.001
elav/RNAi ♀	55.7	45.4	26.7	<0.001	0.63	<0.001	0.034
elav/+ ♀	61.6	45.1	24.4	<0.001	0.10	<0.001	0.013
+/RNAi(2) ♀	60.5	51.0	21.1	<0.001	0.74	<0.001	<0.001

Table A2. Proportion of flies freezing by the end of the experiment (between strains)

	1 vs 1		3 vs 3		5 vs 5	
Strain 1 vs Strain 2 (♀)	Proportions (%)	Chi-square adjusted p	Proportions (%)	Chi-square adjusted p	Proportions (%)	Chi-square adjusted p
CS vs y^1w^{67c33}	64.7 vs 53.8	0.81	43.3 vs 33.3	0.97	20.0 vs 16.0	1
Amontillado vs CS	46.5 vs 64.7	0.053	44.8 vs 43.3	1	45.0 vs 20.0	0.001
Amontillado vs y^1w^{67c33}	46.5 vs 53.8	1	44.8 vs 33.3	0.72	45.0 vs 16.0	0.002
FMRF vs CS	45.8 vs 64.7	0.045	3.9 vs 43.3	< 0.001	0 vs 20	< 0.001
FMRF vs y^1w^{67c33}	45.8 vs 53.8	1	3.9 vs 33.3	< 0.001	0 vs 16.0	< 0.001
FMRF/Def vs FMRF/+	73.1 vs 77.4	1	10.0 vs 30.1	0.004	0 vs 7.7	0.06
FMRF/Def vs +/Def	73.1 vs 60.2	0.26	10.0 vs 35.4	<0.001	0 vs 6.7	0.11
FMRF/+ vs +/Def	77.3 vs 60.2	0.052	30.1 vs 35.4	1	7.7 vs 6.7	1
Nsyb/RNAi vs Nsyb/+	27.9 vs 79.3	< 0.001	7.0 vs 70.0	<0.001	1.8 vs 61.8	<0.001
Nsyb/RNAi vs +/RNAi	27.9 vs 77.6	< 0.001	7.0 vs 41.7	<0.001	1.8 vs 7.3	1
Nsyb/+ vs +/RNAi	79.3 vs 77.6	1	70.0 vs 41.7	0.010	61.8 vs 7.3	<0.001
elav/RNAi vs elav/+	55.7 vs 61.6	1	45.4 vs 45.1	1	26.7 vs 24.4	1
elav/RNAi vs +/RNAi	55.7 vs 60.5	1	45.4 vs 51.0	1	26.7 vs 21.1	1
elav/+ vs +/RNAi	61.6 vs 60.5	1	45.1 vs 51.0	1	24.4 vs 21.1	1

F. Collisions and freezing offset in FMRF mutants

FMRF mutants exhibited considerably less collisions than wild-type flies (mdn G3 = 44 vs 24, $p < 0.001$; G5 = 77.5 vs 45.5, $p < 0.001$, Kruskal-Wallis test). On median, only 15% of all events of freezing offset by female flies in groups of 5 were immediately preceded by a collision with another animal (in groups of 3 the median proportion was 9%). As with wild-type flies, we did not find a negative association between the proportion of freezing offset by collisions and total time spent freezing (fig 4; G3: $r = 0.09$, $p = 0.38$; G5: $r = 0.006$, $p = 0.95$, Pearson coefficient). Similar results were obtained for males (data not shown). The reduction in the number of collisions was not further elaborated in the main work because it was absent in the FMRF Deficiency line, which suggests that it may not be truly connected to activity of the FMRF gene.

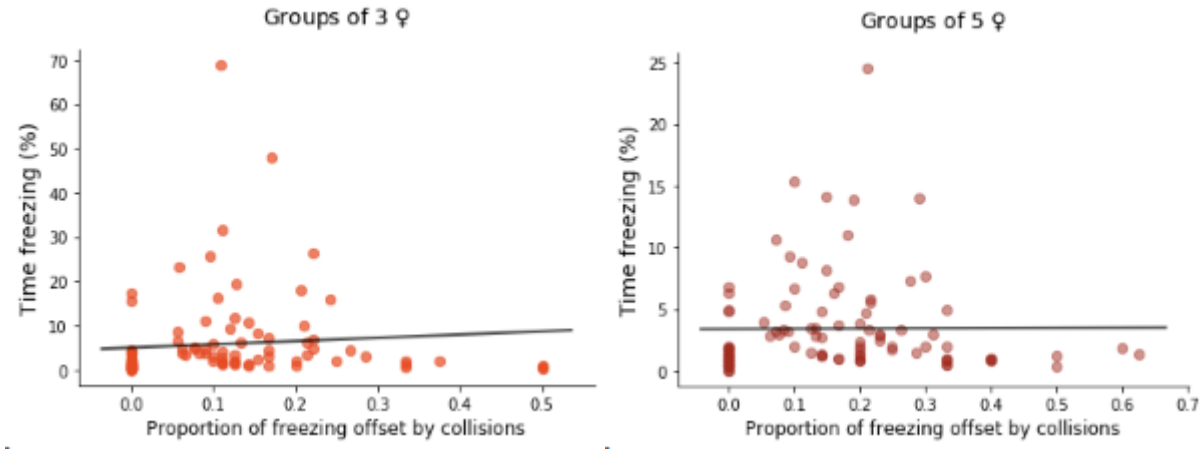

Figure A3. Female FMRF flies — Time spent freezing as a function of the proportion of freezing offset events caused by collisions. Black line represents the linear regression. **A:** Flies in groups of 3. **B:** Flies in groups of 5. Sample sizes ⊂ [100, 102].