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# Behavioural and neurogenetic evidence for emotion primitives in the fruit fly *Drosophila*

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**Emotions, defined as transient states preparing organisms for adaptive responses, are thought to be based on evolutionarily conserved building blocks ("emotion primitives") that are present across mammalian and other vertebrate species. Whether and to which extent these building blocks are found in insects is largely undefined. In this study, we employ the open field test in fruit flies and focus on wall-following and total walking activity as behavioural indicators of emotion-like states.**
**Wildtype and transgenic flies were subjected to various conditions, including social isolation, starvation, and exposure to anxiolytic substances prior to behavioural testing in the open field test. The results indicate that wall-following and total walking activity are modulated by these conditions, with generally increased wall-following observed under aversive stimuli and decreased values under positive conditions. Notably, the behaviour was consistent across different times of the day and independent of circadian rhythms. Genetic manipulation of neuromodulatory systems, such as serotonin, dopamine, and neuropeptide F, supports the role of these pathways in modulating emotion-like states in the fruit fly. Activation of reward-related neurons decreased wall-following, while inhibition increased it, aligning with known effects in mammalian models. Additionally, pharmacological treatments with ethanol and diazepam produced predictable changes in wall-following and total walking activity, reinforcing the validity of the open field test as a measure of emotion-like states in the fly.**
**The findings suggest that *Drosophila* exhibits core emotion-like states, with wall-following and total walking activity serving as reliable indicators of emotional valence and arousal. Our results promote the use of *Drosophila* as a powerful genetic model to dissect the neuronal and neurochemical substrates of emotion primitives, shedding light on the evolution of basic emotional processing mechanisms.**

***Keywords*: emotion primitives, neurochemical brain state, open field, serotonin, diazepam, social deprivation**

## Introduction

Emotions can be defined as transient states that, upon specific events or stimuli, prepare the human organism for adaptive behavioural and physiological responses (Anderson and Adolphs 2014). These states generally involve some form of subjective experience ("feelings") (James 1884). One example is fear, which triggers specific neuronal and autonomic responses that prime the organism for a range of defensive behaviours, such as freezing and fight or flight, depending on the intensity of the threat stimulus and the likelihood of harm (Roelofs 2017). Threat situations and coupled stimulus-dependent defensive behaviour are encountered by all animals, including humans. It is therefore plausible to assume that basal neuronal circuits and neurochemical brain states underlying fear and other basic emotions in humans are evolutionarily conserved in animals (LeDoux 2012; Anderson and Adolphs 2014), even though it is questionable and difficult to test whether non-human animals experience emotions and hence conscious feelings. Thus, contemporary concepts of emotions and increasing evidence suggest that basal building blocks in form of neuronal and bodily states underlying emotions in humans ("emotional processing" (LeDoux 2012), "emotion primitives" (Anderson and Adolphs 2014)) can also be found in animals (Damasio 1999; de Waal 2011; LeDoux 2012; Anderson and Adolphs 2014; Zych and Gogolla 2021). These concepts allow the use of animal models, including genetic model organisms such as the mouse, to dissect the neuronal underpinnings of these basal circuits and brain-body states, which are still poorly defined.

Potentially, *Drosophila* may serve as a valuable model for investigating the neuronal and physiological mechanisms of "emotion primitives" (Anderson and Adolphs 2014; Perry and Baciadonna 2017; Gu et al. 2019) due to its sophisticated genetic tools and available whole-brain connectomes (Scheffer et al. 2020; Schlegel et al. 2023). In fact, a range of fly studies have already been conducted to elucidate neuronal mechanisms and neuromodulators underlying specific emotion-related behaviours like aggression (e.g. (Hoyer et al. 2008; Zhou et al. 2008; Williams et al. 2014; Asahina et al. 2014; Jung et al. 2020), or fear-like defensive behaviour (e.g. (Gibson et al. 2015; Mohammad et al. 2016; Zacarias et al. 2018)). Moreover, evidence of negative cognitive bias, a

characteristic of specific affective states, has been reported for another insect, the honey bee (Bateson et al. 2011).

A widely used test for negative (defensive) emotion-like states in rodents is the open field test (OFT). Typically, animals are placed inside an open arena, and the ratio of the time spent in the central vs. peripheral zone is measured and used to assess fear-like behaviour. Increased aversion of the center – commonly referred to as thigmotaxis- is usually interpreted as a sign of an increased anxiety/fear-like defensive state (Walsh and Cummins 1976; Simon et al. 1994; Belzung and Griebel 2001; Crusio 2001). Similarly, humans with elevated anxiety sensitivity or patients with anxiety disorders show increased time spent along the green wall of a natural open field (Walz et al. 2016). Outside of a fear-related context, the OFT is used to study exploratory behaviour (e.g. (Easton et al. 2003)) and pain-related thigmotaxis (Zhang et al. 2023).

Similar to rodents, fruit flies show increased center avoidance and wall-following (WAFO) in larger open field arenas (Götz and Biesinger 1985; Besson and Martin 2005; Liu et al. 2007; Valente et al. 2007; Soibam et al. 2012; Mohammad et al. 2016), which at least in part represents exploration of the boundary rather than thigmotaxis (Soibam et al. 2012). A pioneering study from the Claridge-Chang laboratory employed a small open field arena to provide evidence for ancient fear-related pathways that are conserved between mice and flies (Mohammad et al. 2016). Specifically, they found that manipulation of serotonin signaling, anxiolytic treatment with diazepam as well as stress produced remarkably similar effects in the OFT as in mice. Thus, *Drosophila* wall following resembled the characteristics of anxiety-related behaviour in rodents. These results suggest that OFT behaviour can be used as a behavioural proxy of a fear-like state in flies.

These findings made us wonder whether WAFO and locomotor activity in the OFT can be used as more general behavioural proxies for emotion-like states beyond fear in *Drosophila*. To investigate this, we subjected different wildtype and transgenic flies to a number of different negative and positive conditions and treatments prior to automated behavioural tracking in the OFT. Then, we quantified total locomotor activity and wall-following behaviour. Our results show that the OFT response of *Drosophila* is remarkably similar to different forms of aversive stimuli and can be reversed by restoration of non-aversive conditions, or positively influenced by thermogenetic activation of appetitive modulatory neurons. We further show that the behaviour in the open field is independent of the time of the day and circadian locomotor activity but differs between flies under laboratory versus natural selection. Collectively, our results suggest that the behaviour in a small open field arena can be used to behaviourally assess core emotion-like states of flies. This opens the possibility to use the OFT as an easy and automatable behavioural readout in conjunction with the sophisticated genetic toolbox and the connectome knowledge of *Drosophila* to study the neuronal and neurochemical substrates of "emotion primitives".

## Results

### *WAFO and TOWA reversibly correlate with aversive signals in Drosophila*

When flies are placed into a larger open field arena, they show a high initial locomotor activity which is strongly dependent on the diameter of the arena and declines quickly over the first minutes to reach a steady activity level (Connolly 1967; Liu et al. 2007). Importantly, Liu et al. (2007) found that this initial activity is completely lost in a circular arena of 6.6 cm diameter, while it was measurable over 3 minutes in larger arenas of 17.6 and 28.5 cm diameter. To reduce the complexity of the open field behaviour (Martin 2003), we therefore used an array of circular arenas with a small diameter (1cm), which we expected to minimise the initial exploratory activity component (Meehan and Wilson 1987) and hence emphasizes a measurement of stimulated activity. Importantly, a 1cm square arena had successfully been used before to study "fear-like" defensive behaviour in the fly (Mohammad et al. 2016). For the current experiments, we put a single male fly into each arena and automatically tracked the behaviour for 10 min, as initial experiments based on 1h recordings revealed that the average activity within this time period is stable and is not significantly different from the following 10 min periods. We then calculated the distance to the center as a measure for WAFO, and the total walking distance (TOWA) as a measure for locomotor activity.

In a first set of experiments, we tested 7d old unmanipulated and unconditioned male flies in the arena, which walked close to the wall, but also crossed the center (Fig. 1A). We then removed the flies from the arena and kept them socially isolated under starvation on pure agarose on a vibrating shaker (420 rpm) for one day. Afterwards, we observed a significantly increased WAFO and TOWA when retested in the OFT (Fig.1A-A'). The males were then placed together as a group in a fresh vial on standard full medium without vibration, and 20 virgin females were added. After one day of restoration, the male flies were retested in the OFT and showed restored pre-stress WAFO and TOWA values (Fig.1 A-A').

When plotted in a two-dimensional coordinate system, the multiple stress experience shifted the data points (X=WAFO, Y=TOWA) for the individual flies towards significantly higher values (Fig.1A'). These data points shifted back towards the pre-stress values after the flies were restored from stress (Fig.1A'). To better visualise the behavioural changes, we normalised the individual values for WAFO and TOWA to the median pre-stress values. We then plotted the resulting relative median values for the whole group, with the X-Y axis representing the respective fold change of WAFO/TOWA compared to the pre-stressed condition (Fig. 1A''). We further reversed the sign of the fold change of WAFO to better reflect that center preference decreases with increasing strength of aversive signals. Due to the normalisation, the median pre-stress value now centers at the axis origin, and the fold-change of -WAFO and

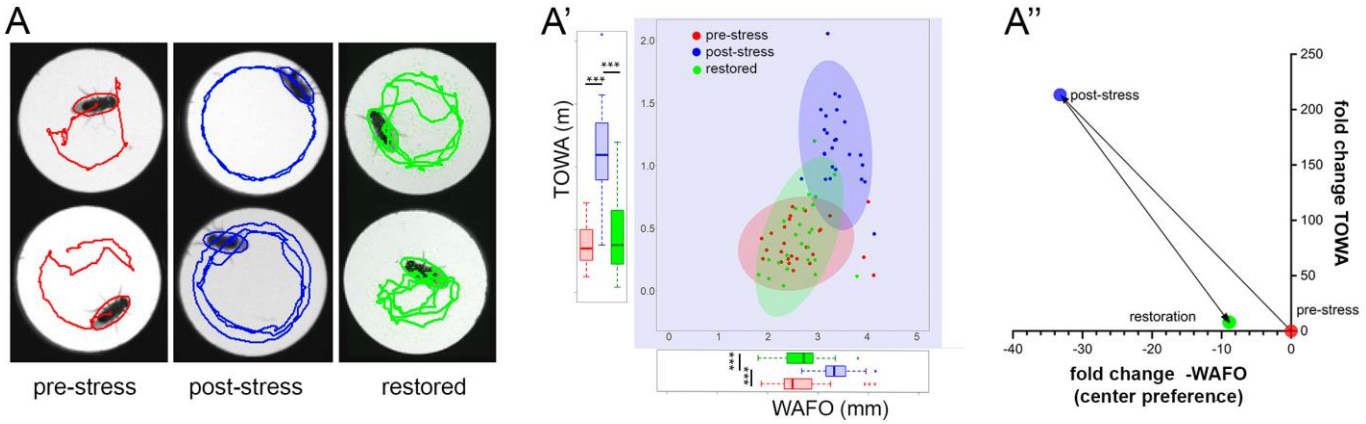


**Figure 1: Wall-following behaviour (WAFO) and total walking activity (TOWA) in the OFT upon exposure to multiple stressors. A)** Typical examples of partial walking trajectories (200 frames, 40 seconds) in the glass-covered arena before experiencing multiple stressors (red), directly after exposure to multiple stressors (blue, one day of social isolation, starvation and sleep deprivation) and after restoration (green, 1 day on food together with other flies of mixed sex and without shaking) as automatically tracked by C-trax. **A')** WAFO and TOWA values increase significantly after experiencing the multiple stressors and decrease back to pre-stress levels after restoration. **A'')** The median values of the data in A') were normalised to the median pre-stress value (red dot) and plotted as fold change compared to the pre-stress levels as the fold change of - WAFO on the X-axis, and fold change in TOWA on the Y-axis.

TOWA can obtain a positive or a negative value (Fig. 1A''). After experiencing stress, the values moved in the direction of an increased WAFO and increased TOWA (Fig. 1A'').

To evaluate the effect of single stressors, seven days old male unconditioned flies were tested pre-treatment on day 1, and then either kept i) isolated (Fig. 2A-A'), ii) starved (Fig. 2B-B'), iii) sleep-deprived (Fig. 2C-C'), iv) sex-deprived as virgins by female rejection (Fig. 2D-D'), or v) sex-deprived after mating in a group without females (Fig. 2E-E') over two to three days (day 2-4) and re-tested for their behaviour in the OFT. At the end, flies were allowed to restore from stress by allowing social contact, feeding, mating and sleep according to the previously exerted stress for one day (day 4 or 5) and then tested again in the OFT. As before, the unconditioned pre-stressed flies defined the baseline value to normalise the results. After social isolation for one and two days (Fig. 2A-A'), and two days of starvation (Fig. 2B-B'), flies showed a significantly increased TOWA. WAFO increased as well after two days of social isolation (Fig. 2A-A'), and after one and two days of starvation (Fig. 2B-B'). These changes in behaviour further increased with four days of social isolation, or two days of starvation (Fig. 1A-B'). The strong increase in TOWA from day 1 to day 2 under starvation can at least partially be explained by increased AKH/octopamine-dependent starvation-induced hyperactivity (Lee and Park 2004; Isabel et al. 2005; Yang et al. 2015; Yu et al. 2016). Upon restoring social contact and providing food, TOWA and WAFO shifted back towards the pre-stress levels which were, however, not fully reached at least for WAFO (Fig. 2A-B'). Similarly, when either sleep-deprived during the day including siesta time (Fig. 2C-C'), or sex-deprived as virgins due to being rejected by mated females for one day (Fig. 2D-D'), flies increased both TOWA and WAFO. These changes became again more pronounced over time with continued sex deprivation like the changes seen after social isolation and starvation (Fig. 2D-D'). In line, extending sleep deprivation into the night resulted in an increase of WAFO, while TOWA decreased possibly due to exhaustion from night sleep deprivation. After restoration, WAFO for both sleep- and sex-deprived flies shifted towards pre-stress levels, while TOWA remained significantly higher compared to the pre-treatment level (Fig. 2C-D'). When mated adult flies were sex-deprived by keeping them in male-only groups, TOWA and WAFO levels (Fig. 2E-E') changed qualitatively like what was observed for sex-deprived virgin males that were rejected by mated females (Fig. 2D-D'). Notably, restoration by mating with a virgin female decreased both WAFO and TOWA (Fig. 2E-E'). One explanation for the difference in TOWA levels between the restored sex-deprived males is that the mating-rejected virgin males (Fig. 2D-D') were sexually naive, while the mated males kept separated from females (Fig. 2E-E') already had the opportunity to mate prior to the test. The increased TOWA levels of the virgin males after first mating during restoration may thus indicate an increased motivation to look for new mating partners due to a novel activation of a neuronal reward circuit compared to mating-experienced males. A previous study in a larger circular arena had not found differences in WAFO between virgin and mated flies in both sexes (Bath et al. 2020), suggesting that the mating status per se has no significant effect on the open field behaviour. Combined, the results for the different single stressors show that stress in general leads to increased TOWA and WAFO levels, while restoration from stress in general reverts both values towards pre-stress levels.

To test for the effect of age, we analysed the open field behaviour of unconditioned young flies 2-3 days, 5-6 days, and 10-11 days after eclosion (under our conditions, flies typically reach an age of 50+ days). We found no effect in TOWA between these age groups (Fig. 2F-F'), indicating that the flies are physiologically of a similar (young) age. In contrast, we observed a significant increase of WAFO in the older groups compared to 2-3 days-old flies (Fig. 2F-F'), which suggests that older flies are more defensive than young flies. Significant differences in WAFO between 5-6- and 10-11 days-old flies were not observed, which validates our standard use of 5-11 days-old flies for the OFT. These age-related changes in WAFO suggest that either age-dependent experience or (brain) maturation during early life alters open field behaviour

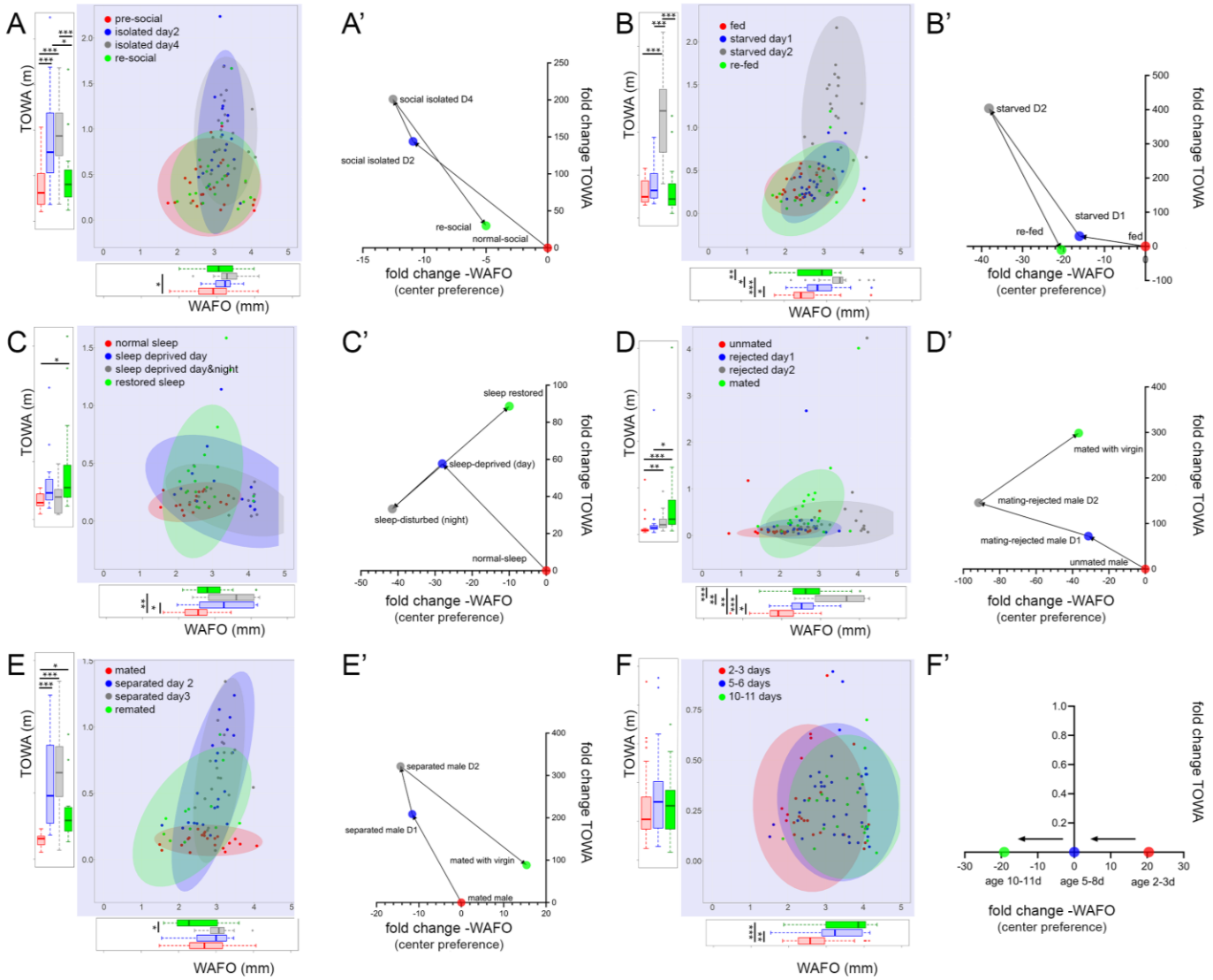


**Figure 2: Wall-following behaviour (WAFO) and total walking activity (TOWA) with different stressors.** Raw data are shown in X), while X') shows the median after normalisation to the pre-treatment condition, plotted as fold changes. **A-A')** Effect of social isolation. **A)** Flies were tested in the OFT, then socially isolated and rested on day two and four, and then regrouped and tested again. Both WAFO and TOWA are strongly affected by social isolation. **A')** With increasing social isolation, flies walk increasingly more and closer to the wall. After restoration of social contact, data points shifted back in direction to pre-stress levels. **B-B')** Effect of starvation. **B)** Flies were tested in the OFT, then starved and tested after one and two days in starvation, and then retested again after one day on food. Both WAFO and TOWA are strongly affected by starvation. **B')** Starvation for one and two days increasingly leads to higher TOWA and WAFO levels. After re-feeding, levels shift back towards pre-starvation levels. **C-C')** Effect of sleep deprivation. **C)** Flies were tested in the OFT, then mechanically sleep deprived and tested in the middle of the light phase (Zeitgeber time (ZT) 7) and at the beginning of the dark phase (ZT13) of day 1 during sleep interruption. Following a day of mechanically undisturbed sleep, flies were then retested. WAFO but not TOWA was strongly affected by sleep deprivation. **C')** Sleep deprivation for the first half or the whole photophase lead to increased WAFO, but had little effect on TOWA. After restoration of sleep, WAFO levels approached pre-stress levels with strongly increased locomotor activity. **D-D')** Effect of mating rejection on unmated males. **D)** Unmated male flies were tested in the OFT, and then paired with mated females. After being mating-rejected from these females for an hour each on day 1-2, male flies increasingly shifted towards TOWA and WAFO levels. After restoration by a successful mating on day 3, a further increase in TOWA but a partial decrease in WAFO was observed. **D')** After being rejected by females on day 1 and 2, virgin males become increasingly active and show increased WAFO. After restoration of courtship, the males showed a further increase in locomotor activity and a decrease in WAFO levels towards the pre-stress condition. **E-E')** Effect of sexual deprivation on mated males. Male flies were mated at day 1 and afterwards tested in the OFT, then sexually deprived and tested on day 2 and 3 of sexual deprivation. This led to a strong and increasing shift towards higher TOWA levels, while WAFO did not change significantly. After restoration by a successful mating on day 3, TOWA levels dropped back and WAFO increased compared to 2 days of sexual deprivation. **F-F')** Effect of age. Different groups of male flies were tested in the OFT at an age of either 2-3, 5-6 and 10-11 days. Aging led to a strong and increasing shift towards higher WAFO levels, while TOWA did not change significantly.

### *Aversive stimuli and anxiolytic substances influence the open field behaviour of flies similar to mice*

We next ask whether aversive stimuli connected to pain in rodents lead to similar behavioural changes in flies. For that, we applied i) a mild mechanical shock (5x tapping) to induce a startle response, ii) a heat pulse of 37°C during the OFT, and iii) 10 brief consecutive electric foot shocks. Flies were tested for their open field behaviour before and during or after the treatment. A mild mechanical shock significantly increased WAFO compared to pre-stimulus conditions (Fig. 3A-A'). This effect was intensified after the mechanical shock was repeated (Fig. 3A-A'), along with a significant increase in TOWA. Similarly, a 37°C heat shock during the OFT increased both WAFO and TOWA (Fig. 3B-B'). Moreover, an aversive electric foot shock (EFS) resulted in increased WAFO and TOWA (Fig. 3C-C'). We next repeated the experiment with EFS with a new set of flies

(Fig. 3D-D'). As before, TOWA was increased, while WAFO reached from medium to elevated levels after EFS (Fig. 3D-D'). The day after, we first retested the flies which showed that WAFO and TOWA were fully restored to pre-shock conditions. Afterwards, we applied 1.2 second EFS. As expected, this resulted in increased WAFO value similar to the EFS on day 1. Strikingly, however, the increase in TOWA was lost after the second EFS (Fig. 3D-D'), reminiscent of the learned "helplessness" state of flies after EFS (Batsching et al. 2016).

The OFT is often used to screen for effects of drugs, including ethanol and diazepam, on pain-induced avoidance and fear-like behaviour in rodent models (Prut and Belzung 2003; Knight et al. 2020; Zhang et al. 2023). To assess whether flies react similarly, we tested the effect of ethanol (which has different dose-dependent effects on locomotor activity in flies (Singh and Heberlein 2000)) and diazepam (a benzodiazepine and prototype anxiolytic drug). Using a capillary feeder (CAFE) assay that allows to quantify food intake (Ja et al. 2007), we first supplied flies for 24h with 10% ethanol mixed with sucrose and then tested flies that had consumed at least 1µl in the OFT. After ethanol consumption, flies showed weakly increased WAFO, and a weakly decreased TOWA compared to controls that consumed sucrose only (Fig. 3E-E'). Thus, feeding on 10% ethanol had a weak sedative but no "anxiolytic" effect on flies under our conditions. Next, we kept flies in vials on 2% coloured agarose mixed with either 4% sucrose (control) or 4% sucrose plus 5 mM diazepam. Food consumption was checked by the appearance of colour in the gut of the flies. Flies that had consumed sucrose plus diazepam-containing food showed a significantly decreased WAFO while TOWA was unaffected compared to controls that had consumed only sucrose (Fig. 3F-F'). This indicates that, unlike 10% ethanol, diazepam has an "anxiolytic-like" effect and confirms that the "anxiolytic" effect of a diazepam-ethanol solution described in an earlier study (Mohammad et al. 2016) was indeed due to the benzodiazepine.

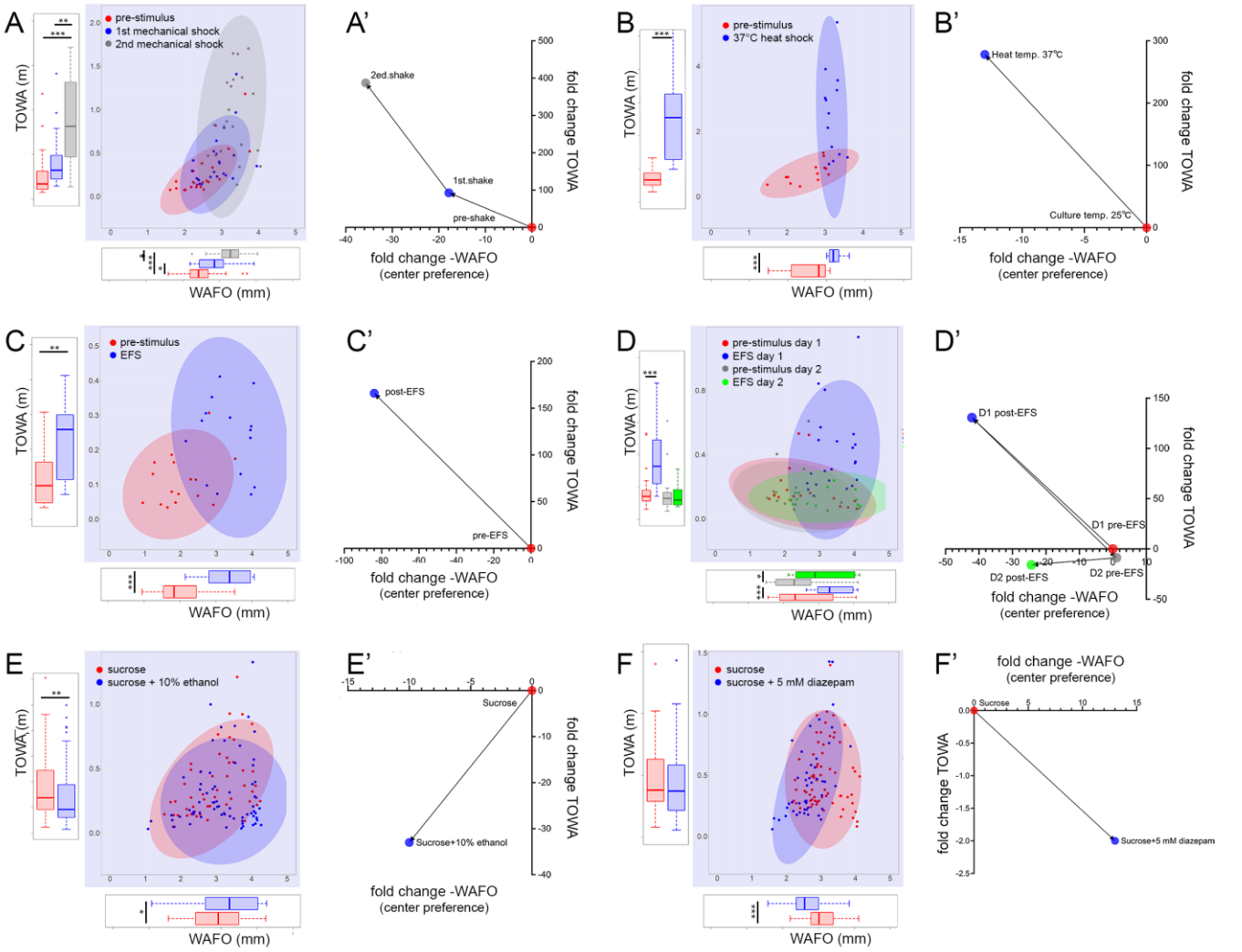


**Figure 3. Aversive stimulation and pharmaceutical treatments consistently alter open field behaviour.** Raw data are shown in X), while X') shows the median after normalisation to the pre-treatment condition, plotted as fold changes. **A-A')** Effect of mechanical shocks (tapping). **B-B')** A 10 min heat-shock of 37°C during the OFT and **C-C')** an electric foot shock (EFS) prior to the OFT increased TOWA and WAFO compared to pre-stimulus conditions. **D-D')** The effect of an EFS given on two consecutive days confirmed the increase of WAFO and TOWA after the first shock, while the second shock had a reduced effect on WAFO and no effect on TOWA. **E-E')** Effect of 10% ethanol. Feeding on 10% ethanol in sucrose induced a mild increase in WAFO compared to sucrose alone, while TOWA was weakly reduced. **F-F')** Effect of 5 mM diazepam. Feeding on 5 mM diazepam plus sucrose induced a stronger increase in WAFO compared to sucrose alone, while TOWA was unaffected.

### Genetic manipulation of neuromodulator signalling affects WAFO rather than TOWA

The results above show that stress and aversive signals trigger a similar open field behaviour in flies as reported for rodents. In mammals including humans, mood, anxiety and fear are under neuromodulatory control by biogenic amines and neuropeptides (Rana et al. 2022; Pourhamzeh et al. 2022). In particular, serotonin (5-hydroxytryptamine, 5-HT), dopamine as well as neuropeptide Y (NPY) signalling are known to strongly affect the open field behaviour of rodents ((Simon et al. 1994; Broderick and Phelix 1997; Prut and Belzung 2003). We therefore asked whether manipulating homolog neurochemical systems in the fly brain also yield similar effects in the open field behaviour as found in mice. First, we revisited the role of serotonin signalling by repeating previous experiments by Mohammad and colleagues (2016). We conditionally down-regulated (by RNA interference) or overexpressed the *Drosophila* serotonin transporter (dSerT) using a temperature-inducible (29°C) pan-neuronal *nSyb tubGal80$^{ts}$* Gal4 driver line based on the TARGET system (McGuire et al. 2003). Control and experimental flies were pre-incubated at either 18°C or 29°C prior to the OFT assays conducted at 20°C. The pre-incubation temperature itself had no effect on the respective GAL4 driver and UAS responder controls (Fig. 4A-D). Overexpression of dSerT at 29°C significantly reduced WAFO (Fig. 4A) in line with the results of Mohammad et al. (2016). RNAi-mediated down-regulation of *SerT* at 29°C, however, also resulted in reduced WAFO (Fig. 4A), contrary to what was expected and found by Mohammad et al. (2016). TOWA was not significantly affected by either downregulation or overexpression of *SerT* (Suppl. Fig. 1). Directly after the OFT, the flies received an electrical foot shock (EFS) in the arena, and then were tested a second time. This treatment significantly increased both WAFO and TOWA in controls and flies with down-regulated *SerT* expression (Suppl. Fig. 2). Yet, flies with *SerT* overexpression showed a significant increase only for TOWA (Suppl. Fig. 2). Moreover, decreased or increased *SerT* expression at the permissive temperature led to a significantly reduced post-EFS increase in WAFO compared to the non-permissive temperature (Fig. 4B). Next, we used the *GMR58E02-Gal4* line and expressed a temperature-sensitive form of dynamin encoded by *shibire* (Shi$^{ts}$) (Kitamoto 2001) to specifically and conditionally suppress neuronal transmission of the dopaminergic protocerebral anterior medial (PAM) neurons that project prominently to the medial lobe of the mushroom body (Liu et al. 2012). While the PAM neurons are an inhomogeneous group of neurons in adult flies, their *en masse* activation (Liu et al. 2012; Burke et al. 2012) or disinhibition (Yamagata et al. 2021) signals reward, and their thermogenetic or optogenetic activation induces valence-driven behaviour such as attraction or aversion (Aso et al. 2014; Mohammad et al. 2024) . Specific silencing of neuronal transmission at 29°C in PAM neurons significantly increased WAFO compared to the same genotype tested at 18°C (Fig. 4C), while temperature itself had no effect in controls (Fig. 4C). As in the dSerT experiment, we found no change in TOWA after suppression of dopaminergic PAM neuron signalling (Suppl Fig. 3). Next, we activated the PAM neurons thermogenetically at 29°C via the temperature-sensitive channel TRPA1 prior to the OFT. This resulted in a significant decrease of WAFO (Fig. 4C) compared to temperature controls. Interestingly, this was accompanied by a significant decrease of TOWA (Suppl. Fig. 3 ).

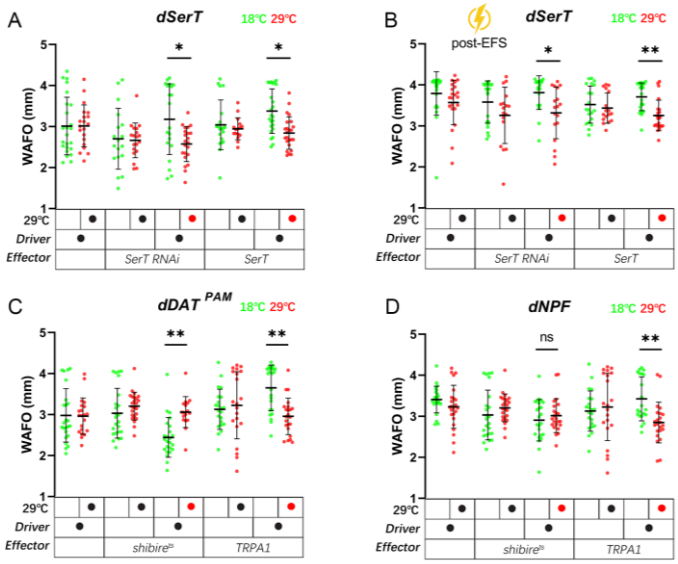


**Figure 4. Genetic manipulation of aminergic and peptidergic signalling affects open field behaviour. A)** Flies with temperature-induced (29 °C, red) conditionally reduced serotonin transporter expression (*nSyb tubGal80$^{ts}$* x *SerT RNAi*) or UAS-mediated SerT overexpression (*nSyb tubGal80$^{ts}$* x *SerT*) showed significantly reduced WAFO compared to flies at the non-inducing condition (18 °C, green), while WAFO was unaffected by temperature in the genetic controls. **B)** Similar experiment as in A) but after an electric foot shock (EFS) showed a reduced but still significant decrease in WAFO after manipulation of SerT expression. **C)** Thermogenetic suppression of dopaminergic reward neurons of the PAM cluster significantly increased WAFO compared to their temperature control, while thermogenetic activation of the same PAM neurons decreased WAFO value compared to their temperature control. Genetic controls were not significantly affected by temperature. **D)** Thermogenetic activation of reward-mediating NPF-positive neurons significantly decreased WAFO compared to temperature controls, while thermogenetic silencing had no effect. Temperature did not significantly affect WAFO in the genetic controls.

Mammalian NPY as well as its fly homolog neuropeptide F (NPF) are key peptides in the regulation of feeding and mating motivation, as well as reward (Krashes et al. 2009; Nässel and Wegener 2011; Loh et al. 2015; Comeras et al. 2019; Ryvkin et al. 2024). In mammals, NPY signalling has an inhibitory effect on fear and anxiety in order to facilitate foraging and other rewarding behaviours (van den Burg and Stoop 2019; Comeras et al. 2019). In flies, thermogenetic activation of NPF neurons is perceived as rewarding as shown by olfactory conditioning (Shohat-Ophir et al. 2012). Moreover, mating-rejected isolated males show significantly decreased NPF transcript and peptide levels compared to virgin or mated controls (Shohat-Ophir et al. 2012). Therefore, we first blocked neuronal transmission specifically in the NPF neurons using *shi$^{ts}$*, yet this did neither affect WAFO nor TOWA (Fig. 4D, Suppl. Fig. 4). Thermogenetic activation of NPF-positive neurons via TRPA1 led to a significant decrease of WAFO (Fig. 4D) but did not alter TOWA (Suppl Fig. 4).

Collectively, these findings show that the open field behaviour of *Drosophila* is influenced by neuromodulators for which the mammalian homologues are known to effect emotional states and open field behaviour.

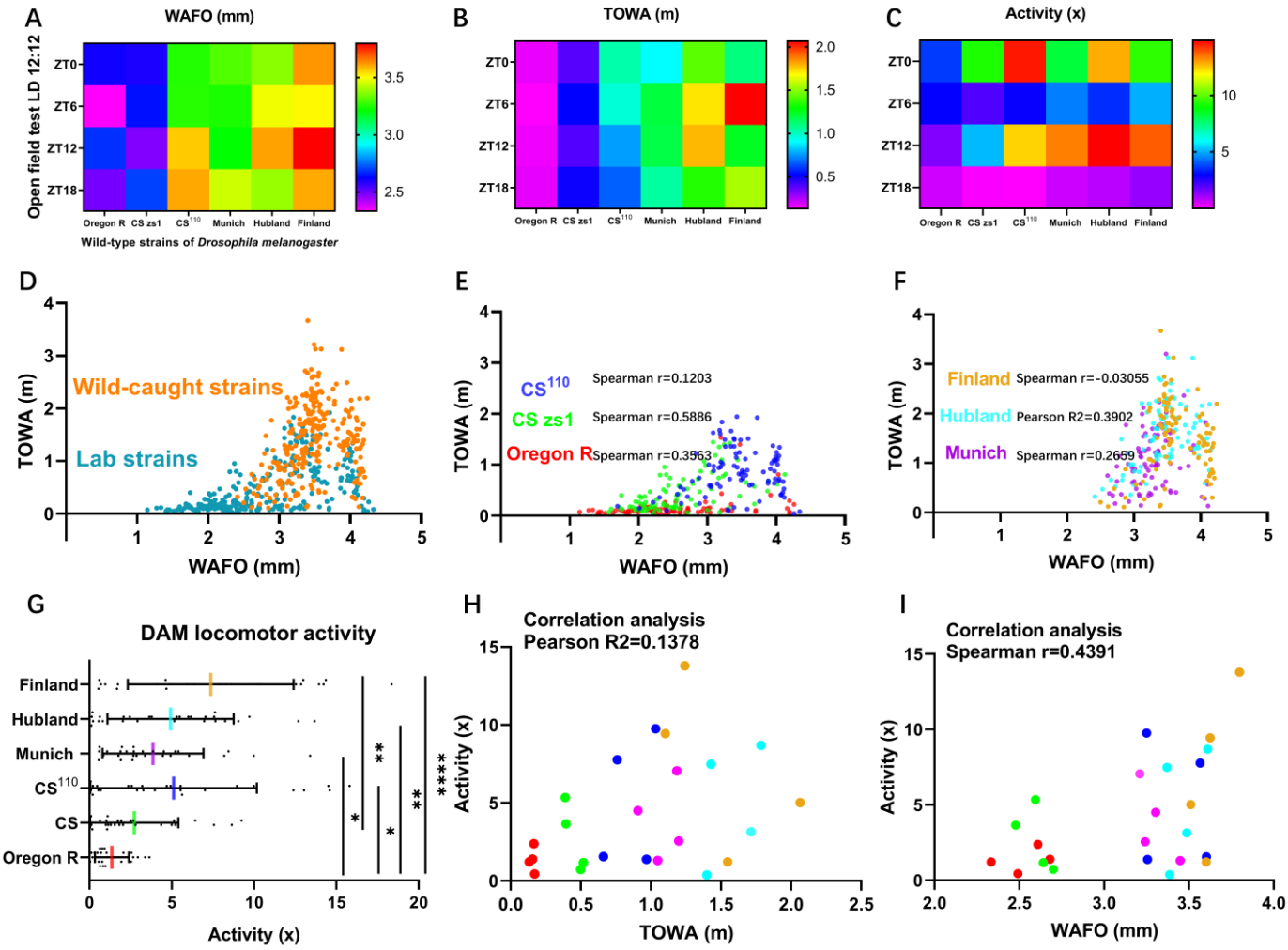


**Figure 5. Comparison of WAFO and TOWA and spontaneous activity between laboratory (OrR, CS, CS$^{110}$) and wild-caught *Drosophila melanogaster* wild-type strains. A)** Heat map for WAFO values obtained in 6h intervals during the day. WAFO appeared to be much lower in the inbred lab strains (CS, OrR) compared to an outcrossed lab strain (CS$^{110}$) and wild-caught strains. **B)** Heat map for TOWA values obtained in 6h intervals during the day. TOWA appeared to be much lower in the inbred lab strains compared to an outcrossed lab strain (CS$^{110}$) and wild-caught strains. **C)** Heat map of spontaneous locomotor activity as measured in the TriKinetics DAM system, obtained in 6h intervals during the day. Spontaneous locomotor activity appeared lower in the inbred lab strains compared to an outcrossed lab strain (CS$^{110}$) and wild-caught strains. **D)** Scatter plot of the WAFO and TOWA values obtained in the laboratory strains (CS, CS$^{110}$, OrR) and the wild-caught strains. Very low WAFO values were only observed in the laboratory strains. **E)** The inbred laboratory strains CS and OrR but not the outcrossed CS$^{110}$ strain showed a weak linear relationship and positive correlation between TOWA and WAFO. **F)** In the wild-caught strains, WAFO and TOWA were not (Finland, Munich) or only weakly (Hubland) positively correlated. **G)** Spontaneous locomotor activity in the DAM monitors was generally higher in the wild-caught strains than in the laboratory-derived strains. **H)** The spontaneous locomotor activity in the DAM monitors was not significantly correlated with the TOWA in the OFT. **I)** The spontaneous locomotor activity in the DAM monitors was only weakly and non-monotonously correlated with WAFO in the OFT.

### *In the OFT, wild-caught Drosophila melanogaster strains are more defensive and aroused than laboratory wildtype strains*

The results above show that fruit flies in the OFT react similarly to stressors and impaired neuromodulatory signalling as rodents for which the open field behaviour can be linked to fear-like states. Like other emotions, fear and anxiety are products of evolution, thought to promote the adaptation of behavioural and physiological responses to the environmental condition, e.g., the presence of a predator or other dangerous situations. However, all experiments so far were conducted with fly strains that for many generations are kept in the laboratory without predators, with *ad libitum* access to food, and with extremely limited possibility to spatially escape from aggressors. We therefore wondered to which degree the observed OFT responses are representing naturally selected responses, and compared the unconditioned open field behaviour among three laboratory wild type strains (OregonR (OrR), CantonS strain (CS), and CantonS flies that were outcrossed against a *period*$^{01}$ strain for 110 generations (CS$^{110}$) (Horn et al. 2019)), as well as three wild-caught strains of *Drosophila melanogaster* (originating from Hubland-Würzburg/Germany (Deppisch et al. 2022), as well as from Munich/Germany and Akaa/Finland (coll: John Parsch and Maaria Kankare within the DrosEU consortium (Kapun et al. 2020)). To cover the natural variation within the wild-caught strains, all experiments were conducted with the F1 outcross of isofemale lines from the respective population. To account and test for possible time-dependent effects, an OFT was performed every six hours during a day in a light-dark cycle of 12:12. Daily rhythmicity or significant day-dependent changes in WAFO were not observed in the tested strains (ANOVA, $p > 0.05$, Fig. 5A-B) except for the CS$^{110}$ strain (ANOVA, $p<0.01$). We infer from this that the naïve WAFO behaviour in the OFT is independent of the

time-of-day and the circadian clock. For TOWA, only the Finnish (ANOVA, $p<0.0001$) and the $CS^{110}$ strain (ANOVA, $p<0.05$), but not the other strains showed significant day-dependent differences in TOWA (Fig. 5 A-B, Suppl. Table 1). These differences, however, did not correlate with the typical morning (around Zeitgeber time (ZT) 0) and evening peak (around ZT12) in spontaneous locomotor activity in the DAM assay (Fig. 5C) which is under circadian control (Schlichting and Helfrich-Förster 2015).

Of note, the two inbred lab strains OrR and CS showed significantly lower average WAFO and TOWA values compared to the outcrossed $CS^{110}$ and the wild-caught strains (Kruskal-Wallis with Dunn's multiple comparison, $p< 0.001$, Fig. 5 A-B, D). Moreover, the average spontaneous activity in the DAM monitor was significantly lower in the inbred CS and OrR strains compared to the outcrossed $CS^{110}$ and the wildtype strains (Kruskal-Wallis with Dunn's multiple comparison, $p< 0.0001$, Fig. 5C, G). These results could be explained by adaptation of the inbred laboratory strains to a spatially restricted life in small vials with *ad libitum* availability of food, resulting in reduced general locomotor activity and reduced drive to escape.

WAFO and TOWA were at best only weakly correlated (Fig. 5 D-F). Similarly, TOWA (Pearson $R^2=0.1378$, Fig. 5H) and WAFO (Spearman $r=0.4391$, Fig. 5I) correlated only weakly with the spontaneous activity measured in the DAM monitors. Taken together, these findings suggest that WAFO, TOWA, and spontaneous activity are largely independent variables.

## Discussion

The main aim of this study was to evaluate whether wall-following (WAFO) and total walking activity (TOWA) in the OFT can be used to behaviourally assess core dimensions of emotion-like states in fruit flies. Our results show that conditions and treatments associated with negative valence in humans or other mammals (including social isolation, starvation, sex or sleep deprivation, heat, mechanical disturbance, and electric foot shocks) generally increased WAFO. On the contrary, conditions and treatments associated with positive valence in mammals (including social company, feeding, and relief from stress) generally decreased WAFO. This raises the question whether WAFO can be interpreted as indicating emotional valence in the fly.

### WAFO as an indicator of emotional valence in the fly?

In humans, valence can be defined as an indicator whether something is good or bad/pleasant or unpleasant/helpful or harmful. As such, valence is one key dimension of emotional states, reflecting the organism's capacity for evaluating stimuli or situations (Barrett 2006). In behavioural terms, positive valence leads to approach behaviour (positive taxis), while negative valence results in withdrawal (negative taxis). In T-maze experiments with *Drosophila* (Tully and Quinn 1985), for example, approach or avoidance of an odour learned to be associated with either reward or punishment is extensively used to dissect the neuronal mechanisms underlying appetitive and aversive memory and valence assignment (e.g. (Keene 2004; Perisse et al. 2013; Aso et al. 2014). One outcome of these and similar studies is that distinct subsets of dopaminergic neurons in the fly brain assign and innately encode positive or negative valence of odors or tastes (Owald and Waddell 2015; Siju et al. 2020). Strikingly, optogenetic activation of a large subset of dopaminergic neurons in the paired-anterior-medial (PAM) cluster (targeted by R58E02-Gal 4 (Liu et al. 2012)) that drives appetitive memory to odors was alone sufficient to elicit attraction in a light-dark optogenetic assay (Mohammad et al. 2024). In the OFT, we found that aversion of the center/approaching the wall was caused by stimuli that would have a negative valence in humans, while stimuli that would have a positive valence in humans elicited an opposite response. Moreover, thermogenetic activation of the R58E02 PAM neuron subcluster that drove attraction in a light-dark optogenetic assay (Mohammad et al. 2024) also increased attraction to the center in the OFT, while conditional block of their activity increased WAFO. Thus, WAFO/center preference appears to be a suitable behavioural indicator of emotional valence in the fly.

In the study of human emotions, dimensional theories (Russell 1980; Watson et al. 1999; Posner et al. 2005) have gained considerable influence and provide a conceptual framework that is extendable to the study of animals as they rely on few empirically measurable dimensions (Mendl et al. 2010; Anderson and Adolphs 2014). One of the most widely used dimensional theories is the circumplex model that is based on two bipolar dimensions thought to represent two independent neurophysiological systems: arousal (active/ activated-inactive/ inactivated) and valence (positive/ pleasure-negative/displeasure) (Russell 1980; Posner et al. 2005). Subjective experiences that can be classified by these dimensions are referred to as "core affect", regarded to be a most basic component underlying emotions (Russell 1980, 2003). Core affect represents the "constant stream of transient alterations in an organism's neurophysiological state that represents its immediate relationship to the flow of changing events" (Barrett 2006), i.e. changes in reaction to internal and external stimuli. A great advantage of the circumplex model is that the degree of arousal and valence cannot only be obtained by oral report but also by objective physiological and behavioural measurements. This allows to extend the circumplex model to non-human animals where subjective report is absent but behaviour and physiology can still be measured (Mendl et al. 2010). Based on the conclusion that WAFO can be used as a proxy for valence, we asked whether TOWA can be used as a proxy for arousal, and hence whether the observed OFT behaviour in the fly can be plotted in a circumplex-like manner.

### TOWA as a proxy for exogenously induced arousal in the fly?

Altered locomotor activity is part of an operational definition of arousal (Pfaff and Banavar 2007) and is thought to be a hallmark of emotion states (Zych and Gogolla 2021). In the fly, like in other animals, endogenous processes such as sleep drive, the satiety state, the emotional state or the circadian clock modulate arousal and locomotor activity in similar directions (van Swinderen and Andretic 2003; Van Swinderen and Andretic 2011).

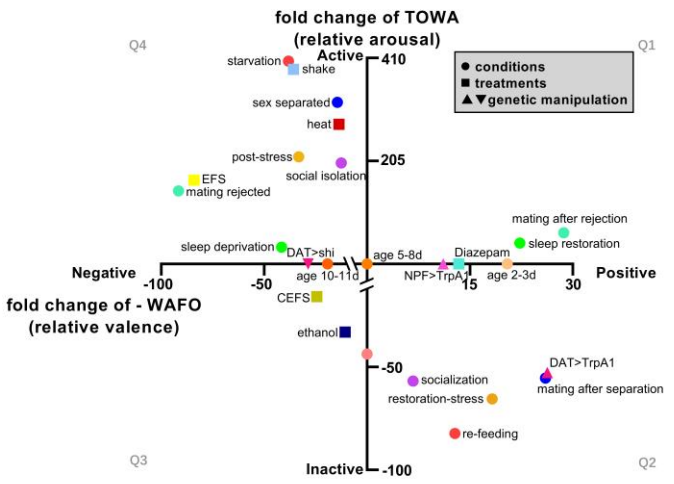


**Figure 6. Summary of the results from all treatments plotted analogously to the circumplex model of valence and arousal.** The median values from all treatments were normalized to the median control value: for post-stress, the pre-stress median served as the control, and for post-restoration, the post-stress median served as the control. These normalized values were then plotted as fold changes relative to the control state, with the fold change in -WAFO represented on the X-axis and the fold change in TOWA represented on the Y-axis. It is important to note that the ratio of the positive and negative axes on the XY plot is not consistent.

Additionaly, external factors such as a new environment can trigger increased sensory and behavioural responsiveness (van Swinderen and Andretic 2003; Van Swinderen and Andretic 2011), for example in the form of increased locomotor activity during exploratory behaviour. We here chose a small and empty OFT arena for our behavioural assay which allowed us to minimise externally-triggered exploratory behaviour (Liu et al. 2007; Soibam et al. 2012). Our results show that TOWA in our OFT setup is not regulated in a circadian or diel manner in various laboratory and nature-derived strains, nor was TOWA significantly correlated with the spontaneous activity level measured in *Drosophila* activity monitors, which is strongly modulated by the circadian clock. In line with an earlier study (Mohammad et al. 2016), we therefore conclude for our OFT setup that TOWA is distinct from spontaneous and clock-regulated locomotor activity. Rather, the observed walking activity appears to primarily represent a startle-like stimulated activity (see (Meehan and Wilson 1987) that is induced by the stimulus of the new environment after the fly is put into the arena (with possible exception of the starvation treatment which is known to induce locomotor hyperactivity (Lee and Park 2004)). Our results show that the magnitude of this stimulated activity depends on the treatment of the fly prior to the new stimulus of the arena, which is in line with the idea of basic emotion-like states in the fly. We assume that this stimulated activity may comprise elements of escape behaviour (one form of defensive behaviour). Interestingly, nature-derived strains showed higher TOWA than the highly inbred lab strains OrR and CS. This may not be surprising as OrR and CS flies have been selected over hundreds of generations for a sedentary lifestyle in fly bottles. In addition, compared to lab strains, nature-derived strains likely possess a higher sensory acuity and may show a higher responsiveness to external stimuli and hence higher arousability. Collectively, our data suggest that TOWA in the OFT can be interpreted as a behavioural proxy for exogenously induced arousal. Moreover, our results showed that WAFO and TOWA are largely independent variables, which corresponds to the orthogonal conceptualisation of valence and arousal in dimensional theories of human emotions

When we next plotted WAFO as an indicator of relative valence (i.e. fold change of - WAFO with respect to the pre-stimulus condition), and TOWA as a proxy of relative arousal (i.e. fold change of TOWA with respect to the pre-stimulus condition) in analogy to the circumplex model (Fig. 6), most conditions and treatments lead to a location in the upper left Q4 quadrant that in humans is associated with conditions like “fearful”, “stressed” or “nervous”, or the lower right Q2 quadrant that in humans is typically associated with states like “relaxed”, “calm” or “contented” (Russell 1980; Posner et al. 2005; Mendl et al. 2010). Few conditions and treatments led to a location in the Q1-Q3 axis. Consecutive foot shocks as well as consumption of 10% ethanol led to a location in Q3 that in humans is associated with conditions like “depressed”, “bored” or “sad”. In comparison to a single electric foot shock that increased WAFO and TOWA, continued foot shock increased WAFO but slightly decreased TOWA. This phenotype is reminiscent of a depressive state in mammals, and resembles the learned helplessness behaviour of flies in the electric shock box in which uncontrollable electric shocks have a longer-lasting negative effect on locomotor activity (Batsching et al. 2016). Mating after rejection and sleep recovery showed the strongest decrease in WAFO with weakly increased TOWA, and hence co-locate with a state that is labeled as “joy” in humans (Q1). We can currently only speculate why most conditions and treatments lead to a location along the Q2-Q4 axis, while only a few lead to a location in the Q1-Q3 with a relatively small difference in TOWA compared to controls. However, we note that, in humans, the negative/inactive quadrant is also frequently underrepresented (Lang et al. 1993; Bradley and Lang 2000; Bradley et al. 2001), possibly because negative emotional states always induce some degree of arousal. Alternatively, the observed distribution may be caused by the nature of the OFT which is typically conceptualised as a behavioural assay for anxiety-like behaviour and thus promotes conditions along the Q2-Q4 axis which have been related to the avoidance of fitness-threatening punishers (Mendl et al. 2010). Moreover, the normalisation against control flies that have their major requirements satisfied (i.e., *ad libitum* access to food, mating and social partners present, optimal temperature and light regime) might negatively bias against emotional states along the Q1 (upper right) and Q3 (lower left) axis which have been related to acquiring fitness-enhancing rewards (Mendl et al. 2010).

Taken together, plotting our WAFO-TOWA data in a circumplex-like system in Fig. 6 produced a distribution that is very similar to the distribution of emotional states in humans in the bipolar valence-arousal circumplex. In addition, the opposite effects of negative and positive stimuli on WAFO are compatible with the “principle of antithesis” (Darwin 1872) that states that opposite

emotional states are bound to opposite behavioural expressions. Anderson and Adolph (2014) functionally defined four features common to different emotions across animals that serve as "building blocks" or "emotion primitives": valence, scalability (intensity), persistence and generalisation. Above, we already argued that WAFO can serve as a proxy for valence. This is supported by a study in *Drosophila* which showed that chronic winning or losing in repetitive aggressive encounters induces internal states that are associated with opposite valence and that affect other, non-aggression related behaviours such as gap-crossing or object exploration (Kim et al. 2018). The increasing changes in TOWA and WAFO upon extended duration of social isolation, sexual deprivation, or mechanical shock suggest that the OFT behaviour is scalable with increasing stimulus strength. Further, the treatments (except for heat) were given prior to the OFT. Thus, the observed behavioural effects in the OFT are in line with the assumption of a short-term persistent emotional-like state in the flies. In addition, different treatments led to a similar behavioural reaction. While this may in part be caused by the nature of the OFT setup, it at least does not speak against the presence of a generalised response. Taken together, these findings lend behavioural support to the presence of basic emotion-like states in the fruit fly.

In mammals including humans, arousal is defined by autonomic nervous system activation with physiological consequences, e.g., sweating, changes in heartbeat, or pupil diameters that ideally are measured along with the behaviour. For example, in mice, measurement of thermal responses allowed to correlate physiological arousal with anxiety-related behavior (Lecorps et al. 2016), and the co-measurement of behaviour and heart rate dynamics allowed to refine and strengthen defensive states (Signoret-Genest et al. 2023). Importantly, changes in heart rate were also found to accompany defense behaviour in tethered *Drosophila* that were presented with a looming threat stimulus (Barrios et al. 2021). Heart rate decelerated during freezing, and accelerated during escape runs and a startle response upon looming (Barrios et al. 2021). A caveat of our study is, that we do not provide physiological evidence for an aroused state since methods to measure heart rate in freely behaving flies are not available yet and were not developed in this study. To increase the validity of our conclusions, further studies are required that measure behaviour in combination with heart rate or other physiological parameters like metabolic rate or tracheal opening (Lehmann 2001).

### Manipulation of neurochemical signalling support the presence of emotion primitives in *Drosophila*

The results obtained by genetic manipulation of neuromodulatory signalling pathways linked to human emotions provide further support for the presence of emotion primitives in the fly. Serotonin signalling has already been shown to influence WAFO in flies and rodents (Prut and Belzung 2003; Mohammad et al. 2016) and presents an ancient fear-related pathway (Mohammad et al. 2016). In both flies and mammals, dopamine and NPY/NPF signalling are associated with the reward system and hence positive valence (see (Dvořáček and Kodrík 2021)). Thermogenetic activation of these neuromodulator systems in the fly decreased WAFO, while inhibition of dopamine signalling increased WAFO.

Confirming a previous study (Mohammad et al. 2016), the anxiolytic GABAA receptor blocker diazepam decreased WAFO, as expected from the anxiolytic effect of diazepam in humans and in agreement with similar experiments in mice (see (Prut and Belzung 2003)).

Interestingly, inhibition of dopamine signalling, activation of NPF signalling and diazepam only affected WAFO, but not TOWA. This suggests the existence of at least partially distinct neuronal substrates for arousal and valence in the fruit fly, in line with the assumption that the two bipolar dimensions arousal and valence in the circumplex represent two independent neurophysiological systems (Russell 1980; Posner et al. 2005)

### Conclusion

Taken together, our results provide behavioural and neurochemical support to the idea that insects such as the fruit fly share stereotypical behavioural phenotypes and evolutionarily conserved and genetically hardwired neuronal circuits ("emotion primitives") that underly basic emotions in humans (Gu et al. 2019). Moreover, the results provide additional support for the use of *Drosophila* as a genetically tractable model to study the neuronal substrate of "emotion primitives", and propose the OFT in a small arena as an easy and automatable behavioural readout. Our study did not attempt to test whether flies have emotions or affective states linked to conscious feelings, nor are the results informative in this respect.

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## Author contributions

Conceptualization: YLW, CW; Methodology: YLW, CW; Formal analysis: YLW, ED, CW; Investigation: YLW, ED, MS; Writing and Visualisation: YLW, CW; Writing review: ED, MS; Funding acquisition: CW.

## Competing interest statement

The authors declare that no competing interests exist.

## Materials and Methods

### Fly strains

*Drosophila melanogaster* strains were cultured at 25°C and 60 % humidity on a 12 h light/ 12 h dark cycle on conventional cornmeal-yeast food. The following wild-type (WT) strains were used: CantonS (CS) Würzburg stock collection, $CS^{110}$ that is CS crossed back to 110 generations to $per^{01}$ (Horn et al. 2019), Oregon R (OrR) Würzburg stock collection, $w^{1118}$ (Bloomington *Drosophila* Stock Center (BDSC)), isogenic nature-derived WT strains Munich/Germany (coll. by John Parsch) and Akaa/Finland (coll. by Maaria Kankare) originated from the DrosEU project (Kapun et al. 2020), WT Hubland was collected close to the Biocenter in Würzburg (Deppisch et al. 2022). For the nature-derived WT strains, we reconstituted the population by outcrossing of isofemale lines. Transgenic/mutant flies used included *tub-Gal80*$^{ts}$, *nSyb-Gal4*, *SerT*$^{GD3824}$ UAS-RNAi (Vienna *Drosophila* Resource

Center (VDRC) #11346), UAS-*SerT* (BDSC #24464), *GMR58E02-Gal4* (*PAM-Gal4,* BDSC #41347 (Liu et al. 2012)), neuropeptide F (NPF)-Gal4 (BDSC #25682), UAS-*TrpA1*, and UAS-*shibire*$^{ts}$.

### Open field test (OFT)

Unless stated otherwise, 5-11 days old male CS flies were sedated on ice, then male flies were picked and individually placed into circular arenas with a diameter of 1 cm arranged in a 4x6 matrix drilled in a 2 mm thick dark-gray plastic plate. The arena plate was sandwiched between two glass plates to prevent flies from escaping, and placed on a white light plate (LED-Panel LP100-NW-Mw (Pollin)) with a light intensity of 270 lm within a climate chamber set to 20°C except for the experiments with *shi*$^{ts}$ flies which were conducted at 29°C. Before starting the recording system (sCMOS USB 3.0 Monochrome DMK 33UX287 camera, ImagingSource, Bremen, Germany), the arena plate was gently shaken to wake up the flies. Fly behaviour was recorded for 10 minutes at 5 frames per second (FPS). The trajectories of moving flies were automatically tracked using Ctrax (Branson et al. 2009). Dead or immobile flies were excluded from the analysis. The XY coordinates from Ctrax were then further analysed using a custom-made Matlab script (developed by YLW and Stefanie Pütz) for total-walking distance (TOWA). Wall-following behaviour (WAFO) was calculated as the distance to the center of the arena as calculated from the XY coordinates.

### Conditions and treatments prior to the OFT

#### Social isolation

After a control OFT, male flies were separated and kept individually in a single vial on food for 4 days without any social interaction, with OFT after day2 and day4 of isolation. Afterwards, the male flies were pooled in one vial and the 20 virgin females were added, followed by a last OFT on the next day.

#### Starvation

After a control OFT, male flies together with females were maintained for 2 days on agarose to provide water but no food source, with OFT after day 1 and 2 of starvation. Afterwards, flies were pooled in one vial containing standard food, followed by a last OFT on the next day.

#### Sleep deprivation

After a control OFT, male flies were placed at ZT3 on a vibrating shaker to prevent sleep. At ZT7 (after siesta) and ZT13 (beginning of the night), flies were re-analysed in the OFT. Afterwards, the need for sleep was restored by placing the flies overnight on a stable surface before the measurement in last OFT.

#### Sex deprivation

1) We used two different sex-deprivation schemes to test for different aspects of lack of courtship in male flies:
Males were collected directly after eclosion, and kept as male-only group for two days to obtain the pre-stress control value. Then, the males were paired for one hour with a similar number of females that had been mated on the previous day, and courtship behaviour was recorded. Males that were rejected by the mated female during that hour were then tested in the open field on the next day. Pairing and rejection was repeated on the next day, followed by a third OFT on the next day. Afterwards, males were pooled in one vial with 20 virgin females, followed by a last OFT on the next day.
2) Males were kept in a mixed-sex group for seven days and then tested in the OFT to obtain the pre-stress control value. Then, the males were separated from females and maintained in a male-only group for 2 days, with OFT after day1 and day2 of separation. Afterwards, males were pooled in one vial with 20 virgin females, followed by a last OFT on the next day.

#### Multiple stressors

After a control OFT, male flies were subjected to a strong stress situation for 24h that combined social isolation, starvation and sleep deprivation as described above, followed by an OFT. Then, the males were pooled in one vial containing normal food without agitation, and 20 virgin females were added, followed by a last OFT on the next day.

#### Mechanical shock

After a control OFT, the arena with the flies was five times sharply tapped on the table edge, directly followed by an OFT. This was followed by a second round of tapping and recording as described.

#### Heat shock

After a control OFT at the standard temperature of 20°C, the arena was placed on a heat plate and heated to 37°C directly followed by a second OFT.

#### Electric foot shock (EFS) and consecutive EFS (CEFS)

*EFS*: after a control OFT, a custom-made plate with an electric grid was inserted between the glass floor and the arena plate. Then, flies received a 90V EFS for 1.2 seconds which was repeated 10x with an interval of 1 minute. The strength of the EFS was strong but not deleterious according to a pre-evaluated response curve. Then, the electric grid was removed and a second OFT was conducted.
*CEFS*: first, male flies were treated with an EFS as described above. Next, the males were transferred back to a vial containing a similar number of females on standard food. On the next day, flies were measured in the OFT, and then received a consecutive EFS, followed by another OFT.

### Pharmaceutical treatments

#### Ethanol

For assessing the effects of ethanol, a Capillary Feeder (CAFE) system (Ja et al. 2007) with 5 µl capillaries was used to provide liquid food containing 10% ethanol, or 5.4% sucrose in water as control. The system consisted of a plate with 24 wells tiled with moist filter paper and closed by a lid was used to house flies individually. Capillaries were inserted into the well through small holes drilled into the lid and an underlying layer of rubber. Test capillaries in wells without flies served to correct for evaporation. On the first training day, the experimental and the control group were fed with freshly prepared food made of 5.4% sucrose in water. The well plate was placed in an incubator at 25 °C and 60 % humidity in a 12 h light/ 12 h dark cycle for approximately 24 hours. On the second day, flies were maintained in the wells, but food-containing capillaries were swapped against water-only filled capillaries for approximately 24 hours to promote hunger. On the third day, the control group was given the same freshly prepared 5 µl 5.4% sucrose water solution as on the training day in the CAFE system. The experimental group received 5 µl 10% ethanol in 5.4% sucrose water solution. Both groups were placed in the incubator for the next 24 hours under the same conditions as above. Finally, flies that at consumed at least 1 µl were analysed in the OFT to assess the ethanol effect on open field behaviour.

#### Diazepam

Male flies were starved overnight in 2% agarose-only vials for 17 hours at 25°C and 60 % humidity on a 12 h light/ 12 h dark cycle. Afterwards, control flies were transferred to a 7ml-volume food vial with 4% sucrose and 2% agarose. The experimental group was maintained in the food vial with 5mM diazepam in 4% sucrose and 2% agarose. A red food dye was added to both foods, and only flies with colored abdomens were collected for conducting a subsequent OFT.

### Thermogenetics

For temperature-controlled expression of *dSerT and dSert-RNAi*, the TARGET system (McGuire et al. 2004) was used, based on the temperature-sensitive driver line *Tub-Gal80*$^{ts}$*, nSyb-Gal4* which was crossed with either *UAS-dSerT* or *UAS-dSert-RNAi* effector lines.
For temperature-controlled inhibition or activation of dopaminergic and NPF neurons, the *GMR58E02-* and the *NPF-Gal4* driver line were crossed to either *UAS-TrpA1* and *UAS-shibire*$^{ts}$ effector lines. TRPA1 is a temperature-dependent cation channel that opens at temperatures around 29°C and induces neuronal activation (Rosenzweig et al. 2005). *shibire*$^{ts}$ is a temperature-sensitive dynamin that loses its function for synaptic vesicle recycling at temperatures around 29°C or higher (Kitamoto 2001).

The newly eclosed progeny 1 of the respective Gal4xUAS crosses were raised at 18°C for 2–4 days prior to the behavioural assays. Then half of the animals were transferred to 29°C overnight (crossing with *UAS-TrpA1* and *UAS-shibire*$^{ts}$) or three consecutive days (crossing with *Tub-Gal80*$^{ts}$*, nSyb-Gal4*) before the OFT, while the other half were kept at 18°C. On the test day, all flies were held at 20°C for a 3h recovery period prior to the assay. Like the animals in experimental group, animals as genetic control raised at identical conditions were generated by crossing the driver line with $w^{1118}$ and the effector line with $w^{1118}$.

### Locomotor activity assay

The locomotor activity of 2-3 days old male post eclosion, was recorded with the *Drosophila* Activity Monitor (DAM) system (Trikinetics, Princeton, MA, USA). 28 flies were placed in separate glass tubes with a diameter of 0.5 cm. The tube was filled on one end with food consisting of 4% sucrose and 2% agar in water. This end is sealed with a rubber plug, while the open

end was blocked by a sponge plug. The activity was recorded for eight days under LD12:12 cycles at 20°C. Data from day 2 to 8 recording were utilized to calculate the mean locomotor activity at ZT0, ZT6, ZT12, and ZT18. To enhance comparability with the OFT, the mean activity was binned in 10-minute intervals during ZT0-1, ZT6-7, ZT12-13, and ZT18-19. This calculation was performed over a one-week period for 28 individual flies, and the results were plotted as activity counts per number of times.

### Data analysis and statistics

Custom-made Matlab scripts were used to analyze the open field behaviour. The arena was seen as a coordinate system with an x- and y-axis, and the coordinates of the center of the arena were defined as:

$$center(X) = \min(X) + \left(\frac{\max(X) - \min(X)}{2}\right),$$

$$center(Y) = \min(Y) + \left(\frac{\max(Y) - \min(Y)}{2}\right)$$

With the instantaneous coordinates of the fly ($X_i$, $Y_i$) and the center coordinates, wall-following (WAFO) of the fly was calculated in pixels per frame by applying the Pythagorean theorem:

WAFO = $\sqrt{(x_i - center(X))^2 + (y_i - center(Y)^2)}$

From this, the median was calculated across all frames.

With the instantaneous coordinates of the fly ($X_i$, $Y_i$) and the coordinates on the next frame (instant location i+1), total walking activity (TOWA) of the fly during the 10min recording was calculated in pixels by applying the summation:

TOWA = $\sum i \sqrt{(x_i - x_{i+1})^2 + (y_i - y_{i+1})^2}$

For the analyses, the median value of the WAFO in each frame during the 10-minutes at 5 fps recording was calculated. According to the ratio between the diameter of the arena in mm and in pixel, WAFO and TOWA were converted from pixel to mm. Flies that did not move all along the wall once were excluded from the analysis as our method does not allow to correctly calculate the center of the arena in that case.

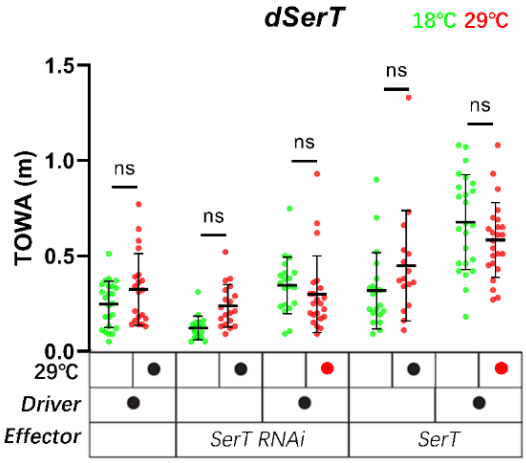
The statistical evaluation and diagrams plotting were carried out by GraphPad PRISM 8.0 (Dotmatics), and by R 4.3.1 (www.r-project.org) using a custom-made script kindly provided by Morgan Oberweiser (University of Greifswald, Germany). For two group comparisons, depending on the data distribution, either an unpaired t-test or a Mann-Whitney U test was used. When more than two groups were compared to each other, either a one-way ANOVA (parametric data) or a Kruskal-Wallis test (non-parametric data) was applied.

## Supplementary table

**Suppl. Table 1: Statistics for Fig. 5A-B.** The data for the different ZT times were analysed by a Kruskal-Wallis test. If significant differences were found, Dunn's multiple comparison test was used for pairwise comparison between ZT times.

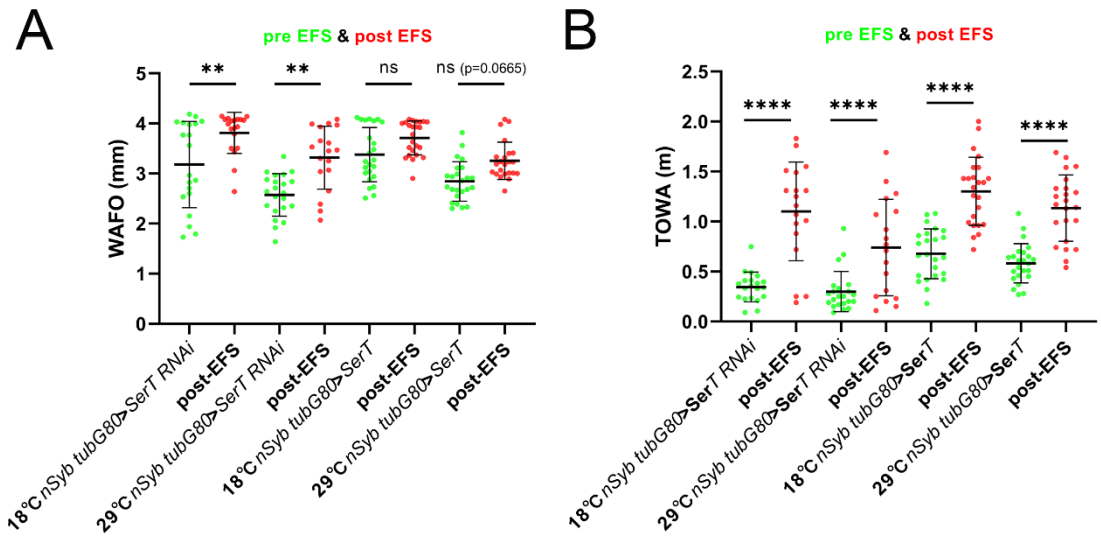
| | Oregon R | | | CS | | | $CS^{110}$ | | | Munich | | | Hubland | | | Finland | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WAFO (mm) | mean | sd | n | mean | sd | n | mean | sd | n | mean | sd | n | mean | sd | n | mean | sd | n |
| ZT0 | 2.61 | 0.71 | 23 | 2.60 | 0.54 | 22 | 3.25 | 0.50 | 24 | 3.30 | 0.38 | 21 | 3.37 | 0.48 | 22 | 3.62 | 0.49 | 24 |
| ZT6 | 2.33 | 0.53 | 22 | 2.64 | 0.56 | 22 | 3.26 | 0.34 | 21 | 3.24 | 0.31 | 22 | 3.49 | 0.31 | 24 | 3.51 | 0.21 | 23 |
| ZT12 | 2.68 | 0.79 | 23 | 2.48 | 0.55 | 22 | 3.57 | 0.53 | 24 | 3.21 | 0.26 | 23 | 3.61 | 0.26 | 22 | 3.80 | 0.41 | 24 |
| ZT18 | 2.49 | 0.86 | 22 | 2.70 | 0.68 | 24 | 3.60 | 0.62 | 24 | 3.45 | 0.38 | 23 | 3.39 | 0.45 | 22 | 3.60 | 0.38 | 23 |
| significance | ns | | | ns | | | ZT6 vs.18: p=0.045 | | | ns | | | ns | | | ns | | |
| TOWA (m) | mean | sd | n | mean | sd | n | mean | sd | n | mean | sd | n | mean | sd | n | mean | sd | n |
| ZT0 | 0.17 | 0.28 | 23 | 0.39 | 0.33 | 22 | 1.03 | 0.53 | 24 | 0.91 | 0.57 | 21 | 1.43 | 0.73 | 22 | 1.10 | 0.63 | 24 |
| ZT6 | 0.13 | 0.06 | 22 | 0.52 | 0.35 | 22 | 0.97 | 0.41 | 21 | 1.20 | 0.54 | 22 | 1.71 | 0.59 | 24 | 2.07 | 0.61 | 23 |
| ZT12 | 0.16 | 0.16 | 23 | 0.39 | 0.37 | 22 | 0.76 | 0.34 | 24 | 1.19 | 0.70 | 23 | 1.79 | 0.54 | 22 | 1.24 | 0.61 | 24 |
| ZT18 | 0.17 | 0.31 | 22 | 0.50 | 0.39 | 24 | 0.66 | 0.47 | 24 | 1.05 | 0.53 | 23 | 1.34 | 0.62 | 22 | 1.55 | 0.70 | 23 |
| significance | ns | | | ns | | | ZT0vs.ZT18: p=0.024 | | | ns | | | ns | | | ZT0vsZT6: p<0.0001<br>ZT0vs.ZT6: p=0.002<br>ZT0vs.ZT18: p=0.037 | | |

## Supplementary figures



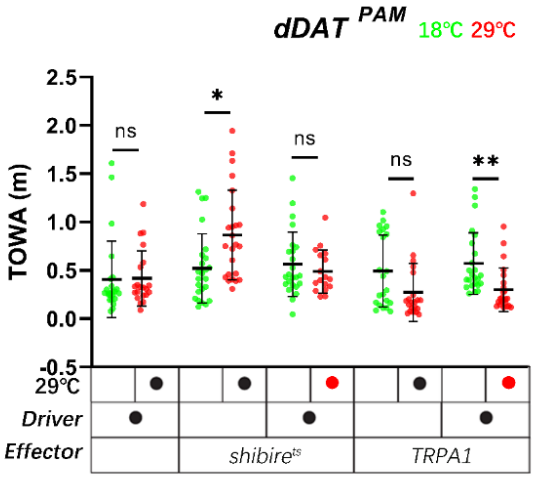

**Suppl. Fig. 1. Genetic manipulation of serotonin signalling has no effect on open field behaviour.**

Flies with temperature-induced (29 °C, red) conditionally reduced serotonin transporter expression (*nSyb tubGal80$^{ts}$* x *SerT RNAi*) or UAS-mediated SerT overexpression (*nSyb tubGal80$^{ts}$* x *SerT*) showed no change in TOWA compared to flies at the non-inducing condition (18 °C, green)



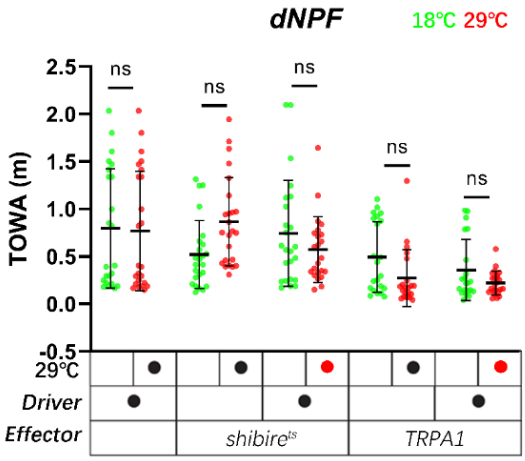

**Suppl. Fig 2. Electric foot shock (EFS) affects open field behaviour in flies with altered *SerT* expression.**

**A)** Flies with reduced serotonin transporter expression (*nSyb tubGal80$^{ts}$* x *SerT RNAi*) cultured at 18°C and 29°C before receiving EFS (green) and after receiving EFS (red) showed a significant difference in WAFO. Flies with UAS-mediated SerT overexpression (*nSyb tubGal80$^{ts}$* x *SerT*) showed a trend of increased WAFO after receiving EFS. **B)** Flies with different transgenic backgrounds (*nSyb tubGal80$^{ts}$* x *SerT RNAi* and *nSyb tubGal80$^{ts}$* x *SerT*) cultured at 18°C and 29°C all showed a significant increased TOWA after receiving EFS (red) compared to the state before receiving EFS (green).




**Suppl. Fig 3. Manipulation of the activity of dopaminergic reward neurons (PAM cluster) affects open field behaviour.**

Thermogenetic suppression of dopaminergic reward neurons of the PAM cluster had no change in TOWA compared to their temperature control. Thermogenetic activation of the same PAM neurons decreased TOWA value compared to their temperature control. Genetic controls were not significantly affected by temperature except the thermogenetic suppression at 18°C (PAM x *shibire*$^{ts}$).




**Suppl. Fig 4. Manipulation of NPF-positive neurons has no effects on open field behaviour.**

Thermogenetic suppression and activation of NPF-positive neurons had no change in TOWA compared to their temperature control.