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RESEARCH ARTICLE

Monoterpenes alter TAR1-driven physiology in *Drosophila* species

Luca Finetti[1], Lasse Tiedemann[2], Xiaoying Zhang[2], Stefano Civolani[1,3], Giovanni Bernacchia[1,*] and Thomas Roeder[2,4,*]

ABSTRACT

Monoterpenes are molecules with insecticide properties whose mechanism of action is, however, not completely elucidated. Furthermore, they seem to be able to modulate the monoaminergic system and several behavioural aspects in insects. In particular, tyramine (TA) and octopamine (OA) and their associated receptors orchestrate physiological processes such as feeding, locomotion and metabolism. Here, we show that monoterpenes not only act as biopesticides in *Drosophila* species but also can cause complex behavioural alterations that require functional type 1 tyramine receptors (TAR1s). Variations in metabolic traits as well as locomotory activity were evaluated in both *Drosophila suzukii* and *Drosophila melanogaster* after treatment with three monoterpenes. A TAR1-defective *D. melanogaster* strain (*TAR1*^{PL00408}) was used to better understand the relationships between the receptor and monoterpene-related behavioural changes. Immunohistochemistry analysis revealed that, in the *D. melanogaster* brain, TAR1 appeared to be mainly expressed in the pars intercerebralis, lateral horn, olfactory and optic lobes and suboesophageal ganglion lobes. In comparison to wild-type *D. melanogaster*, the *TAR1*^{PL00408} flies showed a phenotype characterized by higher triglyceride levels and food intake as well as lower locomotory activity. The monoterpenes, tested at sublethal concentrations, were able to induce a downregulation of the *TAR1* coding gene in both *Drosophila* species. Furthermore, monoterpenes also altered the behaviour in wild-type *D. suzukii* and *D. melanogaster* 24 h after continuous monoterpene exposure. Interestingly, they were ineffective in modifying the physiological performance of TAR1-defective flies. In conclusion, it appears that monoterpenes not only act as biopesticides for *Drosophila* but also can interfere with *Drosophila* behaviour and metabolism in a TAR1-dependent fashion.

KEY WORDS: *Drosophila*, Monoterpenes, Tyramine receptor, Metabolism, Behaviour

INTRODUCTION

Drosophila suzukii (Matsumura) (Diptera: Drosophilidae), commonly known as the spotted wing *Drosophila*, is one of the few Drosophilidae that can lay its eggs on healthy fruits before they become fully ripe (Walsh et al., 2011; Lee et al., 2011). *Drosophila suzukii* is able to infest most fruit and vine species worldwide, with a particular preference for small fruits (Rota-Stabelli et al., 2013). This species causes serious damage to the horticultural economy especially in South-East Asia and its presence has recently also been reported in North America and Europe (Asplen et al., 2015). Moreover, *D. suzukii* can spread rapidly (7–15 generations per year) and has a remarkable ability to adapt to different climatic conditions and host plants (Cini et al., 2012). Chemical pesticides are the main *D. suzukii* control agents, but they need frequent reapplication because of the numerous generations that occur during one crop season. However, repetitive treatments may increase resistance development and have a negative impact on beneficial insects (Desneux et al., 2007; Haviland and Beers, 2012). Alternative and more sustainable control strategies are constantly under investigation (Schetelig et al., 2018). Currently, research on the biology, genetics and physiology of *D. suzukii* has gained interest in order to develop new tools for more effective and environmentally sensitive pest management. Essential oils (EOs) as botanical pesticides are among the most promising pest control methods for future applications. In fact, studies performed in the last decade showed that pesticides based on plant EOs and their constituents (terpenes) are effective against a large number of insects (Bakkali et al., 2008; Isman, 2020). Members of the Drosophilidae family, *D. suzukii* included, are particularly sensitive to EO-based pesticides (Park et al., 2016; Kim et al., 2016; Zhang et al., 2016; Dam et al., 2019). Most EOs are complex mixtures of two predominant classes of molecules, terpenes and phenylpropanoids (Regnault-Roger et al., 2012). Although it is clear that EOs have toxic effects against pest insects, their mechanism of action is still unclear (Blenau et al., 2011; Jankowska et al., 2018). Typically, they are able to reduce or disrupt insect growth at several life stages (Konstantopoulou et al., 1992). It has been shown that terpenes can interact with P450 cytochromes, which are involved in insecticide detoxification processes (Jensen et al., 2006; Liao et al., 2016). Some monoterpenes, for example thymol, may induce neuronal degeneration through a direct interaction with GABA receptors (Priestley et al., 2003) or via acetylcholinesterase inhibition (Houghton et al., 2006; Park et al., 2016). Moreover, monoterpenes might interact with the octopamine/tyramine system, analogous to the adrenergic system present in the vertebrates (Enan, 2001; Kostyukovsky et al., 2002; Enan, 2005a,b; Price and Berry, 2006; Gross et al., 2017; Finetti et al., 2020).

In insects, the main biogenic amines are dopamine (DA), serotonin (5-HT), octopamine (OA) and tyramine (TA). Together, they control and modulate a broad range of biological functions essential for the insect's life (Roeder et al., 2003). The insect nervous system contains high levels of OA and TA, suggesting a role as neurotransmitters (Ohta and Ozoe, 2014), but also as neuromodulators and neurohormones in a wide variety of physiological processes (Pauls et al., 2018).

[1]Department of Life Sciences and Biotechnology, University of Ferrara, 44121 Ferrara, Italy. [2]Laboratory of Molecular Physiology, Department of Zoology, Kiel University, 24098 Kiel, Germany. [3]InnovaRicerca s.r.l. Monestirolo, 44124 Ferrara, Italy. [4]German Center for Lung Research (DZL), Airway Research Center North (ARCN), 24098 Kiel, Germany.

*Authors for correspondence (bhg@unife.it; troeder@zoologie.uni-kiel.de)

G.B., 0000-0002-3259-7273; T.R., 0000-0002-3489-3834

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Originally, TA was considered only as an intermediate product necessary for the synthesis of OA. Nevertheless, today it is known that TA and OA perform important functions independently of each other (Roeder, 2005; Lange, 2009; Roeder, 2020). TA triggers its physiological effects by interacting with and activating the corresponding receptors, belonging to the G protein-coupled receptor (GPCR) family (Evans and Maqueira, 2005). Tyramine receptors (TARs) play important roles in modulating the biology, physiology and behaviour of invertebrates (Ohta and Ozoe, 2014). In fact, either the inhibition or the over-stimulation of TARs can lead to the death of the insect as well as interfere with physical fitness and reproductive capacity (Audsley and Down, 2015). These receptors are classified into two main groups based on their structure and activity: tyramine receptor type 1 (TA/OA or TAR1) and tyramine receptor types 2 and 3 on the other (TAR2 and TAR3) (Wu et al., 2014). TAR1 transcript localization provides clues to its physiological roles. In *D. melanogaster*, the receptor is highly expressed in the central nervous system (CNS; Saudou et al., 1990; El-Kholy et al., 2015). A similar expression pattern has also been observed in *D. suzukii*, *Rhodnius prolixus*, *Chilo suppressalis*, *Plutella xylostella*, *Mamestra brassicae* and *Agrotis ipsilon*, suggesting a crucial role for TA as neuromodulator and neurotransmitter (Wu et al., 2013; Hana and Lange, 2017; Ma et al., 2019; Brigaud et al., 2009; Duportets et al., 2010; Finetti et al., 2020). Several studies have reported the importance of TA, through its interaction with TARs, in a variety of processes including olfaction, reproduction, flight, locomotion and metabolic traits (Lange, 2009; Neckameyer and Leal, 2017; Roeder, 2020). In particular, TA appears to play a role in locomotor modulation (Saraswati et al., 2004; Hardie et al., 2007; Rillich et al., 2013; Schützler et al., 2019), egg-laying behaviour (Donini and Lange, 2004; Fuchs et al., 2014), sex pheromone production (Hirashima et al., 2007), metabolic traits including the regulation of energy expenditure (Brembs et al., 2007) and hormone release (Roeder, 2020). Despite the physiological importance of TA in invertebrates, little is known about TARs. In 2000, Kutsukake and co-workers characterized *D. melanogaster honoka*, a mutant line with an impaired TAR1, exhibiting a different behaviour towards repellent odours. Furthermore, Li et al. (2017) have shown that TAR1-deficient flies exhibit significant changes in metabolic control such as higher body fat, lower starvation resistance and movement activity. Similar TAR1-mediated metabolic alterations were observed by Ishida and Ozaki (2011) in starved flies. Nevertheless, the existence of crosstalk between the tyraminergic system and other systems, such as the octopaminergic and dopaminergic systems, makes it difficult to precisely dissect the physiological processes controlled by TA (Li et al., 2016).

In the last few years, several studies have suggested that TAR1 might be an interesting target for insecticides, specifically for bioinsecticides. For example, monoterpenes appear to be able to interact with TAR1 directly. In particular, Enan (2005b) was the first to describe an agonistic effect of several monoterpenes (thymol, carvacrol, α-terpineol and eugenol) on *D. melanogaster* TAR1. However, the same monoterpenes did not show this pharmacological profile with *D. suzukii* and *Rhipicephalus microplus* TAR1. They acted instead as positive allosteric modulators, increasing the potency of TA activity (Gross et al., 2017; Finetti et al., 2020). Furthermore, a recent study from our lab has described a possible molecular mechanism underlying the toxicity of these molecules towards insects (Finetti et al., 2020). In particular, the observed downregulation of *D. suzukii* TAR1 (DsTAR1) after monoterpene exposure might represent a compensatory mechanism in response to enhanced receptor signalling due to the positive allosteric modulatory effect of monoterpenes on the receptor.

The current study presents a detailed investigation on *D. suzukii* behaviour upon monoterpene treatment, in order to understand whether *DsTAR1* downregulation could affect fitness and physiology. Furthermore, a TAR1-defective *D. melanogaster* line was used as a control to compare the effects of chronic TAR1 impairment on *D. melanogaster* physiology with monoterpene treatment of *D. suzukii* flies.

MATERIALS AND METHODS

Fly stocks

Drosophila suzukii were kindly provided by the Entomological Laboratory of the Agricultural Sciences Department of the University of Padua (Italy) and maintained on an artificial diet with a 16 h:8 h photoperiod, at a temperature of 22±1°C. *Drosophila melanogaster* mutant lines were as follows: $TAR1^{PL00408}$ was generated by the Gene Disruption Project (Bloomington Stock Center, Bloomington, IN, USA, no. 19486; Bellen et al., 2004) and TAR1-Gal4 was previously created in the Molecular Physiology group from the University of Kiel (El-Kholy et al., 2015). The *D. melanogaster* $TAR1^{PL00408}$ defective line, which showed 50% downregulation of the target gene as confirmed by RT-qPCR (Fig. S1), had already been backcrossed several times with y^1w^{1118}, the control line for all corresponding experiments, as previously described (Li et al., 2016). All *D. melanogaster* flies were raised on standard food at 25±1°C (12 h:12 h light:dark photoperiod).

Fumigant toxicity assay

A glass cylinder (10 cm in height, 4.5 cm inner diameter; 150 ml volume) was employed to calculate the monoterpene median (LC_{50}) and maximum lethal concentration (LC_{90}) values for *D. suzukii* and *D. melanogaster* y^1w^{1118} control and to perform monoterpene exposure. Monoterpenes including thymol, carvacrol and α-terpineol were dissolved in acetone and applied to a piece of filter paper (2 cm×2 cm). The filter paper was placed on the bottom lid of the cylinder, inside a small cage to prevent direct contact of the flies with the monoterpenes. The concentrations ranged between 0.067 and 67 µl l^{-1} and acetone alone was used as a negative control. After CO_2 anaesthetization, 30 flies (15 males and 15 females) were placed inside the cylinder with 1 ml of solid diet. The top and the bottom of the cylinder were sealed with Parafilm and the assay was maintained at 22±1°C for *D. suzukii* or 25±1°C for *D. melanogaster* flies. After 24 h, the flies were collected. For LC_{50} and LC_{90} calculation, at least 100 flies were tested, in four replicates.

Quantitative real-time PCR analysis

Total RNA was extracted from *D. suzukii* or *D. melanogaster* y^1w^{1118} control line adult flies subjected to monoterpene exposure, using an Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA). A 1 µg sample of RNA was treated with DNase I (Thermo Fisher, Waltham, MA, USA) and used for cDNA synthesis, using a OneScript® cDNA Synthesis Kit (Abm, Vancouver, BC, Canada), according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad) in a 12 µl reaction volume containing 1.6 µl cDNA (diluted 1:2), 6 µl SYBR PCR Master Mix (Vazyme, Jiangsu, China), 0.4 µl forward primer (10 µmol l^{-1}), 0.4 µl reverse primer (10 µmol l^{-1}) and 3.6 µl nuclease-free water. Thermal cycling conditions were: 95°C for 2 min, 40 cycles at 95°C for 15 s and 60°C for 20 s. After the cycling protocol, a melting-curve

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Table 1. Primers used in this study

Primer	Sequence (5′–3′)
Dmel_TAR1-Fw	CACTCTGGAGGCGGAAAGT
Dmel_TAR1-Rev	GCAACGGAGTGACAGAAACG
Dmel_Actin-Fw	GCGTCGGTCAATTCAATCTT
Dmel_Actin-Rev	AAGCTGCAACCTCTTCGTCA
Dmel_Tubulin-Fw	TGTCGCGTGTGAAACACTTC
Dmel_Tubulin-Rev	AGCAGGCGTTTCCAATCTG
Dsuz_TAR1-Fw	GCAGTCCTCGTCCACCTG
Dsuz_TAR1-Rev	TTAAGGGACGTCTGCTCGTC
Dsuz_AK-Fw	CTACCACAACGATCCAAGA
Dsuz_AK-Rev	AAGGTCAGGAAGCCGAGA
Dsuz_TBP-Fw	CCACGTGAATCTGTGCT
Dsuz_TBP-Rev	GGAGTCGTCCTCGCTCTT
DmTyrR-Exon1-Fw	CAACTCAAAGCGACAGACCA
DmTyrR-Exon2-Rev	TACATGCGTCTTGGTGGAAA
Rpl32-Fw	CCGCTTCAAGGGACAGTATC
Rpl32-Rev	GACAATCTCCTTGCGCTTCT

analysis from 55 to 95°C was applied. In *D. suzukii*, expression of *TAR1* was normalized using arginine kinase (*AK*) and TATA box protein (*TBP*) genes, which served as reference genes (Zhai et al., 2014). In *D. melanogaster* y^1w^{1118}, expression of *TAR1* was normalized using *actin* and *tubulin* genes that served as reference genes (Ponton et al., 2011). Gene-specific primers (Table 1) were used and four independent biological replicates, made in triplicate, were performed for each sample.

TAR1 immunohistochemistry

A TAR1-Gal4 *Drosophila* line was crossed with a UAS-GFP line in order to visualize the complete brain expression pattern of the receptor. The brains were dissected from F1 flies in cold Schneider's *Drosophila* medium and fixed in 4% (w/v) paraformaldehyde in PBS for 90 min at room temperature. The samples were then washed 3 times in PBST and blocked for 30 min in blocking buffer (1× PBS, 2% NP-40, 10% goat serum) at room temperature. The samples were incubated with primary antibodies in blocking buffer (anti-GFP rabbit 1:300, AB3080, Sigma-Aldrich, St Louis, MO, USA; and anti-Nc82 mouse 1:20, Developmental Studies Hybridoma Bank, University of Iowa) overnight at 4°C and washed 3 times for 5 min in PBST. Subsequently, the samples were incubated with secondary antibodies in blocking buffer (donkey anti-rabbit IgG Alexa Fluor-488 1:300, 711-545-152, Jackson ImmunoResearch, West Grove, PA, USA; and goat anti-mouse IgG Alexa Fluor 555 1:300, 115-165-003, Jackson ImmunoResearch) for 3 h at room temperature and washed twice for 5 min in PBST. Brains were mounted directly on slides and analysed by a Zeiss Axio Imager Z1 microscope equipped with an apotome (Zeiss, Oberkochen, Germany).

Body fat quantification

Total body triglyceride (TG) content was estimated using the TG colorimetric assay kit GPO-PAP method (Elabscience, Wuhan, China). Three flies were accurately weighed and homogenization medium (9 times the volume, 0.1 mol l^{-1} phosphate buffered saline, pH 7.4) was added. The sample was mechanically homogenized on ice with a motorized pestle and centrifuged (at 500 ***g*** for 10 min); 7 µl of the supernatant was added to 700 µl of working solution, thoroughly mixed and incubated for 10 min at 37°C in the dark. Absorbance was read at 510 nm and distilled water, added to 700 µl of working solution, was used as a blank. TG content was estimated using a glycerol solution (2.26 mmol l^{-1}) as standard. Five independent biological replicates were performed for each sex and genotype.

Dye-labelling food intake quantification

Dye-labelling food intake quantification was performed as described by Deshpande and co-workers (2014), with minor modifications. In brief, five flies of each sex and genotype were placed into a vial with 2 ml of 1× dyed medium (2.5% yeast, 2.5% sucrose, 1% agar and 1% Brilliant Blue FCF; Sigma Aldrich). After 2 h of feeding, the flies were collected and frozen at −80°C. Frozen flies were transferred to 1.5 ml Eppendorf tubes, homogenized with a manual pestle in 50 µl of 1% PBST and centrifuged for 1 min at 12,000 ***g*** to clear the debris. The supernatant absorbance was measured at 630 nm on a label-free EnSight Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA). The values obtained from flies fed with non-labelled food were used as a control and subtracted from experimental readings. To determine the dye concentration of each fly homogenate, a standard curve was generated with serial dilutions of an initial 10 µl aliquot of the non-solidified dye-labelled food added to 990 µl of 1% PBST. At least five independent biological replicates were performed for each sex and genotype.

Metabolic rate determination

Metabolic rate was assessed by respirometry as described previously (Yatsenko et al., 2014). In brief, for each sex and genotype, three adult flies were placed in each vial and metabolic rate was measured for 2 h using respirometry. The CO_2 yield during the test was calculated based on the volume (µl) of CO_2 produced per hour per fly. Data were obtained from five independent biological replicates.

Rapid iterative negative geotaxis (RING) assay

The negative geotaxis assay was performed based on a published protocol (Gargano et al., 2005). In brief, five flies of each sex and genotype were placed into a 20 cm-tall glass tube without CO_2 anaesthesia. The tube was tapped twice to move flies to the bottom

Table 2. LC_{50} and LC_{90} of fumigant active monoterpenes thymol, carvacrol and α-terpineol against *Drosophila suzukii* and *Drosophila melanogaster* y^1 w^{1118}

Compound	Slope (±s.e.)	LC_{50} (µl l^{-1})	LC_{90} (µl l^{-1})	χ^2
D. suzukii				
Thymol	1.704±0.318	**1.085** (0.549–1.575)	6.117 (4.362–10.854)	2.605
Carvacrol	2.289±0.341	**0.844** (0.322–1.340)	3.075 (1.930–8.744)	3.991
α-Terpineol	2.647±0.307	**1.494** (0.677–2.446)	4.563 (2.754–14.164)	6.493
D. melanogaster y^1w^{1118}				
Thymol	1.749±0.209	**0.604** (0.152–2.036)	3.260 (1.172–24.484)	3.472
Carvacrol	1.864±0.258	**0.592** (0.156–1.636)	2.888 (1.136–38.072)	2.168
α-Terpineol	1.677±0.433	**0.984** (0.300–1.524)	5.252 (3.080–16.900)	1.343

LC_{50} and LC_{90} values are means and 95% confidence interval. Bold indicates LC_{50} values used in exposure experiments.

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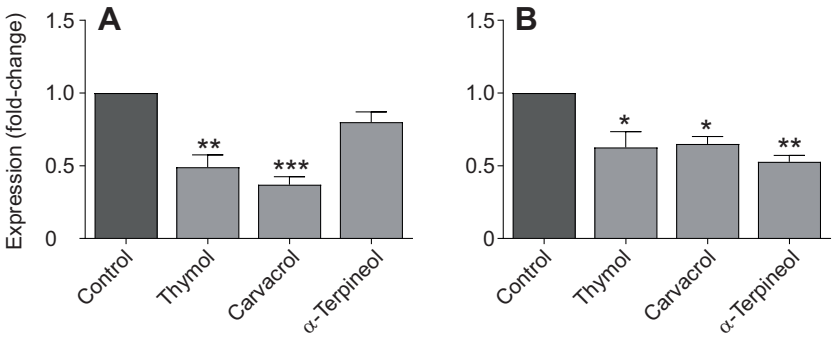

Fig. 1. *TAR1* expression levels after 24 h of continuous exposure to monoterpenes. *Drosophila suzukii* (A) and *D. melanogaster* y^1w^{1118} (B) were exposed to the LC_{50} of thymol, carvacrol and α-terpineol. Data represent means ±s.e.m. of four independent experiments performed in triplicate. **P*<0.05, ***P*<0.01 and ****P*<0.005 versus control according to one-way ANOVA followed by Dunnett's test for multiple comparisons. Arginine kinase (*AK*) and TATA box protein (*TBP*) were used as reference genes in *D. suzukii* analysis (Zhai et al., 2014); *actin* and *tubulin* were used as reference genes in *D. melanogaster* y^1w^{1118} analysis (Ponton et al., 2011).

and the climbing height of flies was photographed after 2 s. The average distance climbed (in cm) for each fly was measured using ImageJ software. Five independent biological replicates per sex and genotype were performed.

Starvation-resistance assay

The starvation resistance assay was performed by placing 25 flies of each sex and genotype into vials containing 1% of agar. The vials were maintained at 22±1°C for *D. suzukii* or 25±1°C for *D. melanogaster*. Dead flies were counted every 2 h until all flies were dead. For each genotype and sex, four independent biological replicates were performed (at least 100 flies).

Statistical analyses

LC_{50} and LC_{90} values were evaluated using POLO-plus software. All statistical analyses were performed using GraphPad Prism software (version 6). All data represent means±s.e.m., evaluated using one-way ANOVA followed by Dunnett's test for multiple comparisons.

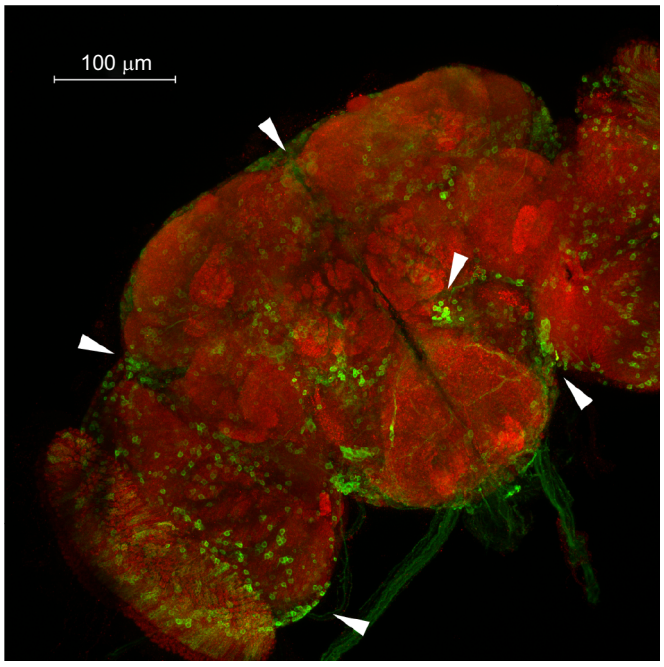

Fig. 2. Activity of the TAR1 promoter in the *D. melanogaster* brain. Representative confocal image of GFP expression driven by TAR1-Gal4: synaptic regions are labelled with the presynaptic marker Nc82 (anti-Bruchpilot; red); TAR1 is marked by anti-GFP antibody. TAR1 is mainly localized in the pars intercerebralis, lateral horn, suboesophageal ganglion, antennal and optic lobes as indicated by white arrowheads. Scale bars: 100 µm.

RESULTS

Monoterpene LC_{50} calculation

The results of the LC_{50} and LC_{90} estimation as obtained by POLO-plus analysis for each monoterpene, performed on both *D. suzukii* and *D. melanogaster* y^1w^{1118} flies, are summarized in Table 2. The table reports the LC_{50} and LC_{90} values, the 95% confidence limits

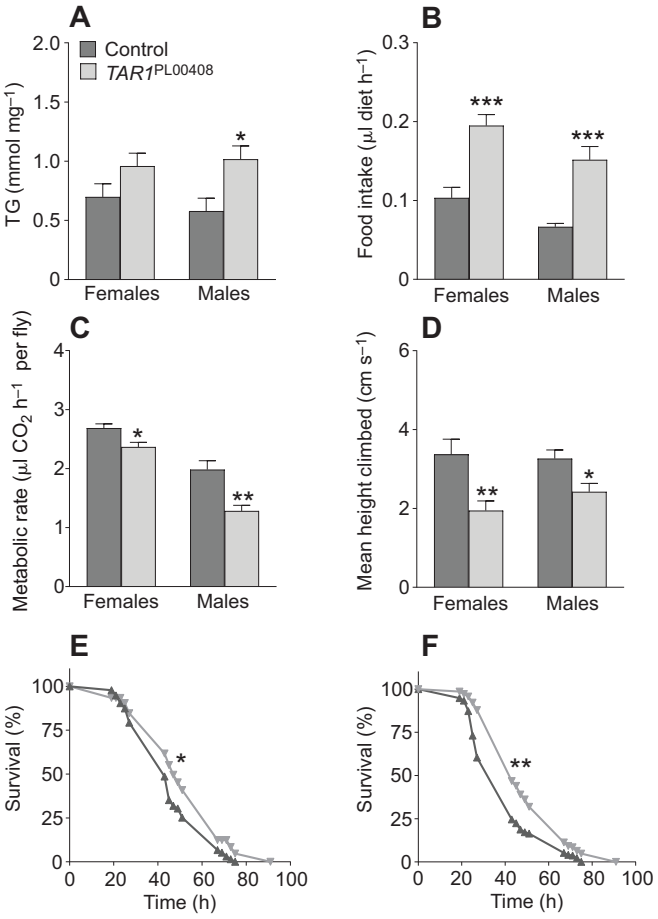

Fig. 3. Physiological, metabolic and behavioural alterations in flies with an impaired TAR1. Total body triglyceride (TG) content (A), food intake (B), metabolic rate (measured as CO_2 production; C), climbing activity (measured by the RING assay; D) and starvation resistance of females (E) and males (F) were tested in control *D. melanogaster* y^1w^{1118} flies and mutant *D. melanogaster TAR1*^{PL00408} flies of both sexes. For all experiments, means ±s.e.m. of at least four independent biological replicates are shown. **P*<0.05, ***P*<0.01 and ****P*<0.005 versus control according to Student's *t*-test. For starvation resistance, statistical analyses were performed using the log-rank test.

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(Robertson et al., 2017), the slopes (angular coefficients) of lines and the values of χ^2 for each monoterpene.

TAR1 expression analysis after monoterpene exposure

To evaluate the effect of exposure to monoterpenes on the expression levels of *TAR1* gene in both *D. suzukii* and *D. melanogaster* y^1w^{1118}, flies were exposed to the respective LC_{50} concentration of thymol, carvacrol and α-terpineol, and mRNA levels were analysed by qPCR. The exposure induced an interesting downregulation of *TAR1* gene expression in both genotypes. In *D. suzukii*, significant differences were observed for thymol and carvacrol (Fig. 1A) but not for α-terpineol. In contrast, in *D. melanogaster* y^1w^{1118}, all three monoterpenes induced a significant downregulation of *TAR1* although this was less marked than that for *D. suzukii* (Fig. 1B).

TAR1 expression in *D. melanogaster* brain

In order to determine the physiological functions controlled by TAR1, the receptor localization in *D. melanogaster* brain was investigated by immunohistochemistry. The Gal4-UAS system was used to follow TAR1 promotor activity with a GFP reporter, then recognized by the anti-GFP antibody.

The receptor showed specific expression in the pars intercerebralis as well as the lateral horn, suboesophageal ganglion, olfactory and optic lobes (Fig. 2A–C), suggesting that TAR1 might be implicated in important physiological traits in *Drosophila*.

Role of TAR1 in *Drosophila* physiology

To elucidate the role of TAR1 in metabolic traits as well as locomotor control and physiological aspects in *Drosophila*, the *D. melanogaster* $TAR1^{PL00408}$ strain was enrolled in several behavioural assays. Despite mutant *D. melanogaster* $TAR1^{PL00408}$ and control *D. melanogaster* y^1w^{1118} flies showing no statistical difference in overall body mass (data not shown), the reduced expression of *TAR1* translated into a higher propensity for TG accumulation in male flies (Fig. 3A) and a greater food intake in both sexes (Fig. 3B). Therefore, $TAR1^{PL00408}$ flies showed higher resistance to starvation than control flies (Fig. 3E,F). These changes were furthermore associated with a slower metabolism in TAR1-impaired insects (Fig. 3C). The increased TG accumulation and the slower metabolism could also be related to the lower propensity to move of the $TAR1^{PL00408}$ flies (Fig. 3D). To test whether monoterpenes, besides downregulating *TAR1*, might also alter the physiology of *D. suzukii* and *D. melanogaster* (wild-type or $TAR1^{PL00408}$ strain), 24 h after the continuous monoterpene LC_{50} exposure, flies were challenged with several behavioural tests, as detailed below.

Effect of monoterpene treatment on total body TG content

Exposure to monoterpenes for 24 h caused a higher TG content in males of both *D. suzukii* and *D. melanogaster* y^1w^{1118} flies as compared with females (Fig. 4A–D). In particular, the TG content was significantly higher upon thymol and carvacrol exposure in *D. suzukii* males only (Fig. 4B), while both *D. melanogaster* y^1w^{1118} females and males showed a significantly higher TG content after carvacrol exposure (Fig. 4C,D). When the same treatments were applied to *D. melanogaster* $TAR1^{PL00408}$ insects, no changes were observed in TG content, which was indistinguishable from that of the untreated control sample (Fig. 4E,F). This evidence would suggest that monoterpenes can induce an increase in total fat deposition that requires TAR1 receptors be functional.

Effect of monoterpene treatment on food intake

Food consumption was quantified after 2 h of feeding on a dye-labelled diet. A significantly higher food intake was observed only after α-terpineol exposure in both *D. suzukii* and *D. melanogaster*

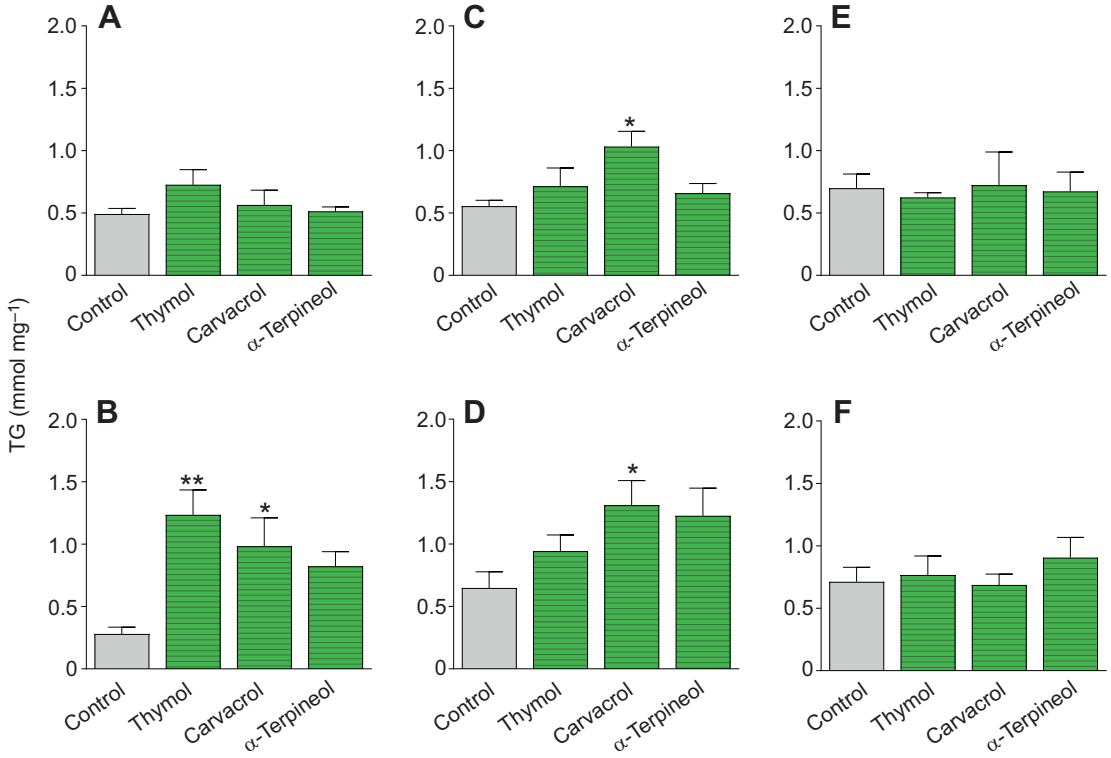

Fig. 4. Total body TG content after 24 h exposure to monoterpenes. TG content of *D. suzukii* (A,B), *D. melanogaster* y^1w^{1118} (C,D) and *D. melanogaster* $TAR1^{PL00408}$ (E,F) females (top) and males (bottom). Data are means±s.e.m. of four independent biological replicates. **P*<0.05 and ***P*<0.01 versus control according to one-way ANOVA followed by Dunnett's test for multiple comparisons.

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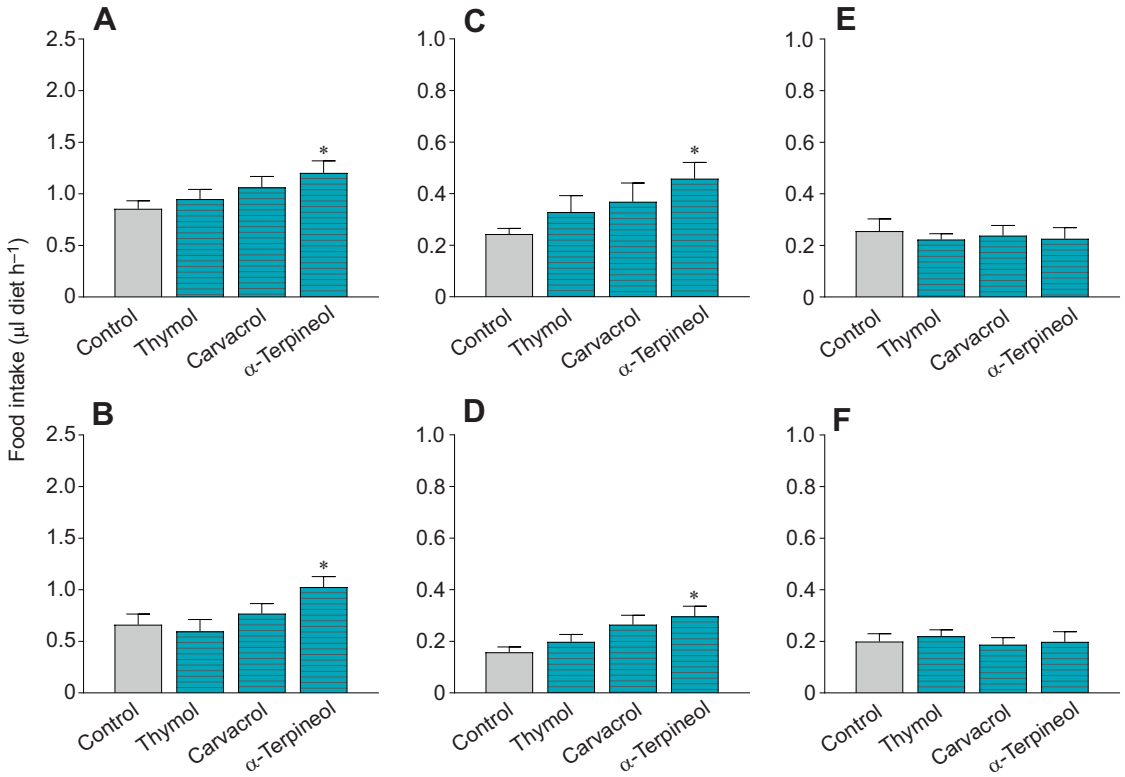

Fig. 5. Food intake after 24 h exposure to monoterpenes. Food intake of *D. suzukii* (A,B), *D. melanogaster* y^1w^{1118} (C,D) and *D. melanogaster TAR1*^{PL00408} (E,F) females (top) and males (bottom). Data are means±s.e.m. of five independent biological replicates. *P<0.05 versus control according to one-way ANOVA followed by Dunnett's test for multiple comparisons.

y^1w^{1118} of both sexes (Fig. 5A–D). The increased food intake might explain the high TG levels observed in *D. suzukii* and *D. melanogaster* y^1w^{1118} of both sexes after monoterpene exposure. However, monoterpene treatment did not cause any change in food consumption in *D. melanogaster TAR1*^{PL00408} mutant flies (Fig. 5E,F), further suggesting the requirement for an active TAR1.

Effect of monoterpene treatment on metabolic rate

In order to determine whether monoterpenes and *TAR1* downregulation affect metabolism, metabolic rate was analysed in all *D. suzukii* and *D. melanogaster* genotypes after treatment with the different monoterpenes. In *D. suzukii*, males, but not females, treated with the three monoterpenes showed a significantly lower metabolic rate than control flies (Fig. 6A,B). Carvacrol and α-terpineol were able to reduce the metabolic rate in *D. melanogaster* y^1w^{1118} males and females (Fig. 6C,D). Conversely, *D. melanogaster TAR1*^{PL00408} metabolic rate appeared to be unaffected by the treatments and was therefore undistinguishable from that of the untreated controls (Fig. 6E,F).

Effect of monoterpene treatment on locomotory activity

The observed metabolic changes in terms of energy expenditure and TG content might also affect the physical activity of flies. Therefore, the ability of flies exposed to monoterpenes to walk upwards on a vertical surface in negative geotaxis was used as a motility behavioural assay. In comparison to untreated controls, *D. suzukii* and *D. melanogaster* y^1w^{1118} males showed a statistically significant reduction in climbing ability after α-terpineol treatment only (Fig. 7B,D). *Drosophila melanogaster* y^1w^{1118} female motility was negatively affected only by thymol (Fig. 7C), while *D. suzukii* females did not respond to the RING assay at all, in both control and treated samples (Fig. 7A). The climbing ability in both *D. melanogaster TAR1*^{PL00408} sexes was unaffected by exposure to monoterpenes (Fig. 7E,F), confirming the hypothesis of TAR1 involvement in this behavioural trait.

Effect of monoterpene treatment on starvation resistance

Finally, a starvation resistance assay was performed to investigate whether the monoterpene-mediated metabolic modifications could affect general fitness. Given the higher food intake and TG content caused by monoterpene treatment, an enhanced resistance to starvation was expected. *Drosophila suzukii* and *D. melanogaster* y^1w^{1118} showed different results depending on the monoterpene used as compared with the control (Fig. 8A–D). According to log-rank statistical analysis, a significant reduction in starvation resistance was detected in *D. suzukii*, for both males and females, after carvacrol treatment (Fig. 8A,B), while both *D. melanogaster* y^1w^{1118} sexes were less resistant to starvation after thymol exposure. Moreover, α-terpineol treatment reduced starvation resistance only in *D. melanogaster* y^1w^{1118} females (Fig. 8C,D). Conversely, carvacrol exposure significantly increased starvation resistance in *D. melanogaster* y^1w^{1118} males (Fig. 8C). *Drosophila melanogaster TAR1*^{PL00408} flies were again unaffected by the treatment, showing starvation resistance comparable to that of controls (Fig. 8E,F).

DISCUSSION

The biogenic amine TA is a mediator of several physiological functions in invertebrates (Roeder, 2005; Lange, 2009), but its mechanism of action is still far from being fully characterized. TA activates intracellular responses by interacting with specific GPCRs:

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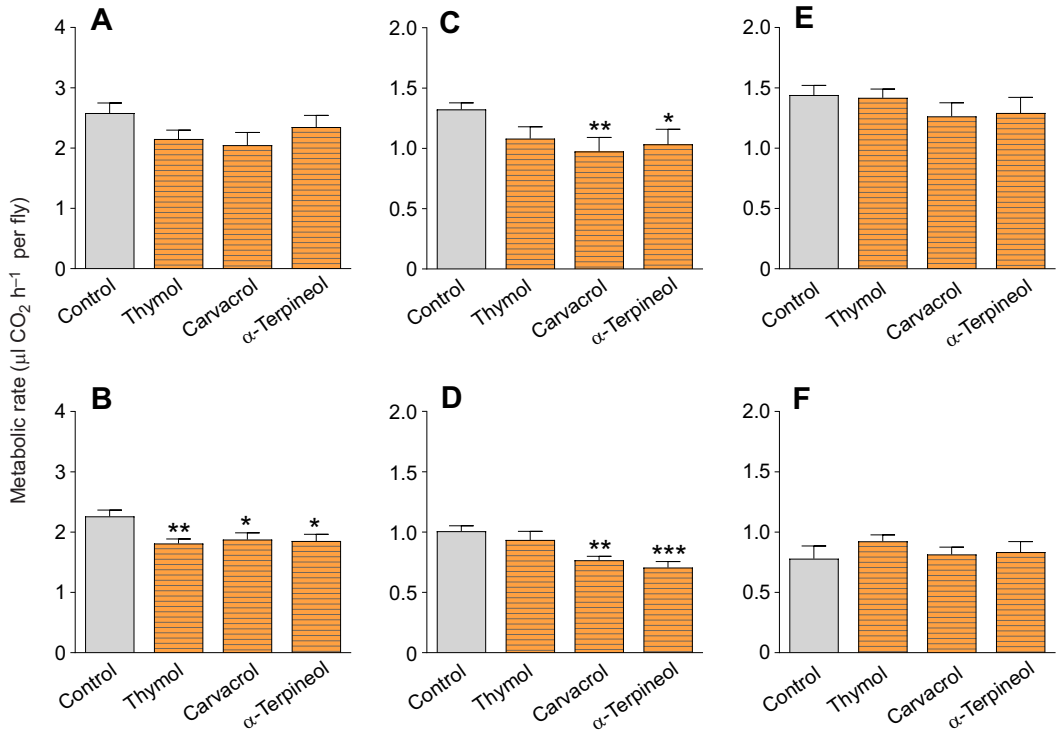

Fig. 6. Metabolic rate after 24 h exposure to monoterpenes. Metabolic rate was measured as CO_2 production in *D. suzukii* (A,B), *D. melanogaster* y^1w^{1118} (C, D) and *D. melanogaster TAR1*^{PL00408} (E,F) females (top) and males (bottom). Data are means±s.e.m. of five independent biological replicates. **P*<0.05, ***P*<0.01 and ****P*<0.005 versus control according to one-way ANOVA followed by Dunnett's test for multiple comparisons.

the tyramine receptors, TARs (Saudou et al., 1990; Roeder et al., 2003). TAR1 is highly expressed in the CNS of numerous insects, thus suggesting its involvement in essential behavioural processes (El-Kholy et al., 2015; Hana and Lange, 2017; Finetti et al., 2020). Furthermore, several studies have suggested that TAR1 is a direct target for biomolecules with insecticidal action, such as

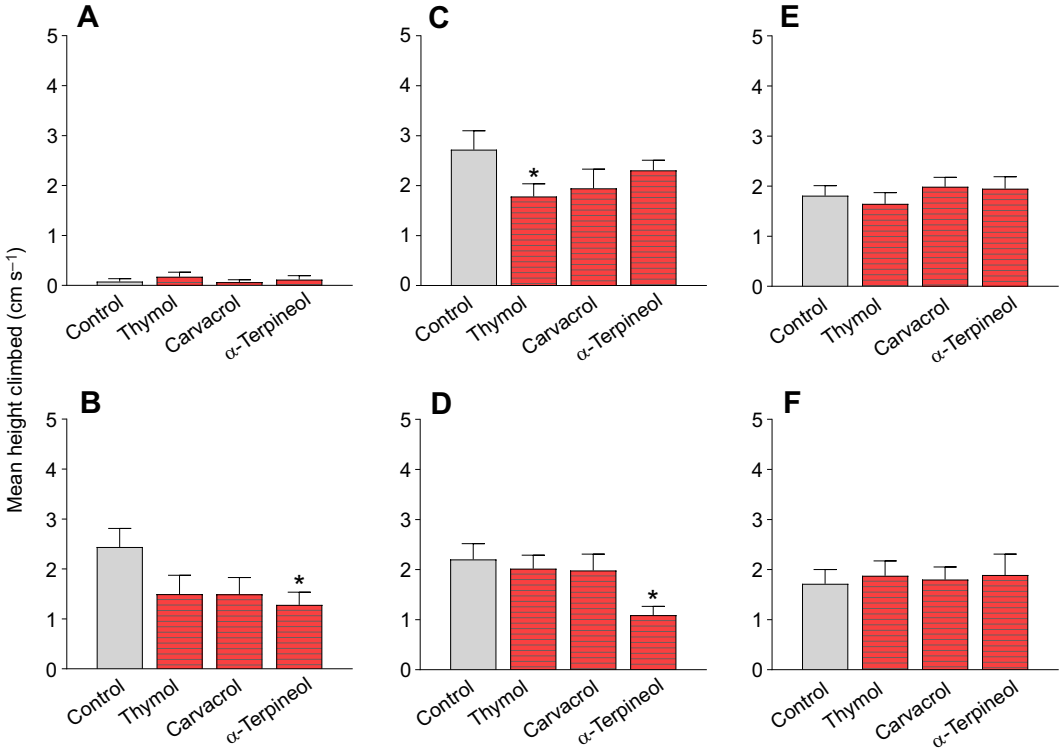

Fig. 7. Mean height climbed after 24 h exposure to monoterpenes. Vertical movement capacity was assessed by the RING assay for *D. suzukii* (A,B), *D. melanogaster* y^1w^{1118} (C,D) and *D. melanogaster TAR1*^{PL00408} (E,F) females (top) and males (bottom). Data are means±s.e.m. of five independent biological replicates. **P*<0.05 versus control according to one-way ANOVA followed by Dunnett's test for multiple comparisons.

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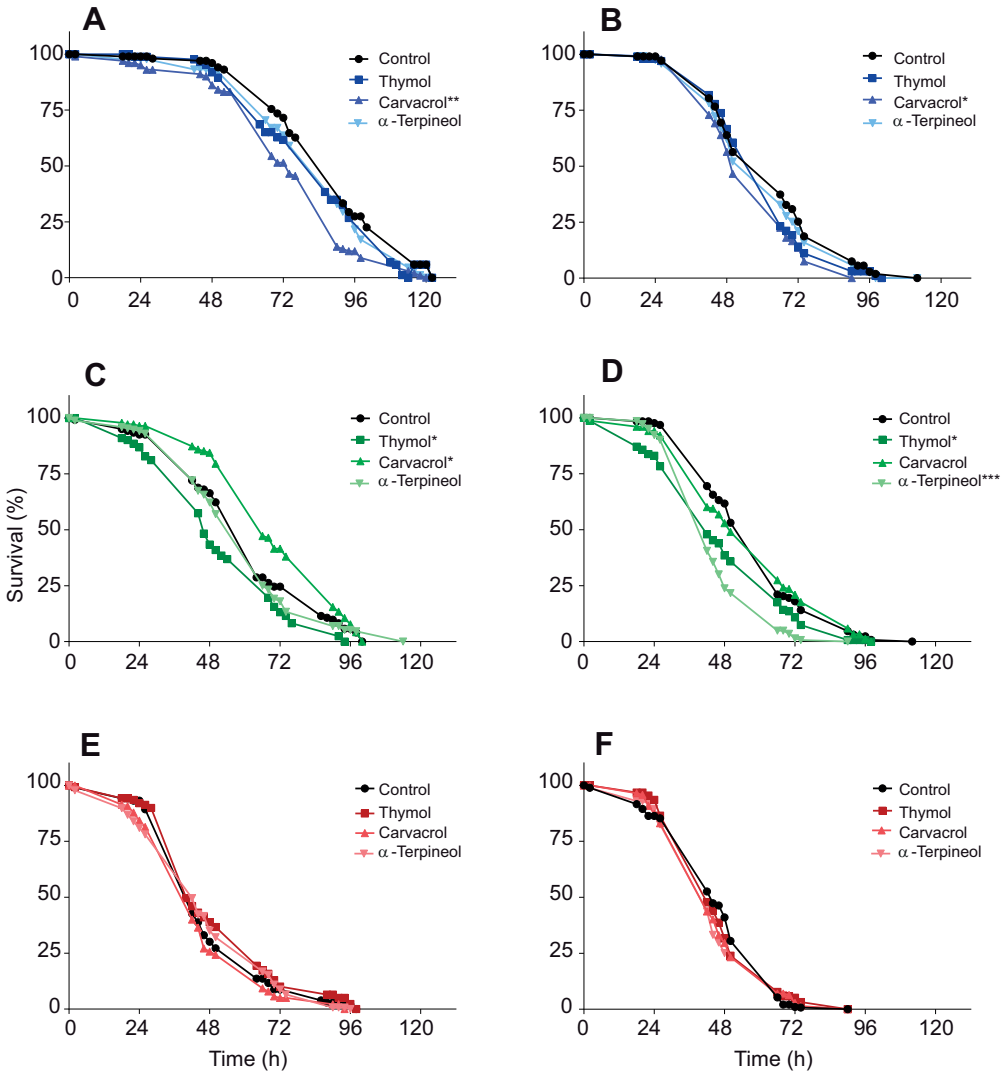

Fig. 8. Starvation resistance after 24 h exposure to monoterpenes. Percentage survival after treatment for *D. suzukii* (A,B), *D. melanogaster* y^1w^{1118} (C,D) and *D. melanogaster* $TAR1^{PL00408}$ (E,F) females (left) and males (right). Five independent biological replicates were performed with the log-rank test statistical analysis. **P*<0.05, ***P*<0.01 and ****P*<0.005 versus control.

monoterpenes. In fact, it has been reported that the *D. melanogaster* and *R. microplus* TAR1, when expressed in a heterologous cell system, respond to the administration of monoterpenes with an increased release of cytosolic calcium (Enan, 2005a; Gross et al., 2017). Recently, the same intracellular response has been observed in our laboratory for *D. suzukii* TAR1, allowing us to hypothesize that the interaction between monoterpene and receptor causes a downregulation of the gene coding for the receptor (Finetti et al., 2020). To further study the effects of the monoterpenes on TAR1 and on the insect physiology, a *D. melanogaster* TAR1-defective line ($TAR1^{PL00408}$) was evaluated together with corresponding controls and *D. suzukii*. Comparative studies using these two *Drosophila* species are possible as they are phylogenetically highly related and their TAR1 share a high degree of homology (98%) (Finetti et al., 2020).

Firstly, the identification of the LC_{50} for the three monoterpenes thymol, carvacrol and α-terpineol, for both *D. suzukii* and *D. melanogaster* y^1w^{1118} via a fumigant assay (Park et al., 2016), revealed that the most toxic monoterpene was carvacrol with an LC_{50} of 0.844 µl l^{-1} for *D. suzukii* and 0.592 µl l^{-1} for *D. melanogaster*. Similarly, Zhang and co-workers (2016) observed that carvacrol was the most toxic monoterpene for *D. melanogaste*r. Interestingly, when $TAR1^{PL00408}$ flies were treated with the monoterpenes at the LC_{50} calculated for the y^1w^{1118} strain, a 40% reduction in mortality was observed as compared with the control (data not shown), suggesting a strong correlation between TAR1 and the insecticidal activity of these monoterpenes. A similar observation was made in a *D. melanogaster* TAR1-deficient strain (specifically $TyrR^{Neo30}$), which appeared to be insensitive to thymol and carvacrol when topically applied (Enan, 2005a).

All three monoterpenes tested, thymol, carvacrol and α-terpineol, by 24 h of fumigant treatment, were able to induce TAR1 downregulation not only in *D. suzukii* (as already established, Finetti et al., 2020) but also in *D. melanogaster*. As TAR1 is mainly expressed in the CNS, the greatest impact of its downregulation might be expected in this region.

As shown by El-Kholy et al. (2015), in a study focused on *D. melanogaster* brain, TAR1 is expressed in the pars intercerebralis, mushroom bodies and ellipsoid body, as also confirmed by Li et al. (2016). Our study revealed that TAR1 is strongly expressed not only in the pars intercerebralis and the mushroom bodies but also in the lateral horn, suboesophageal ganglia and antennae mechanosensory centre. Even if the physiological significance of these specific TAR1 expression patterns in the *Drosophila* CNS is still unclear, they could be connected to the functions associated with the corresponding brain areas. The pars intercerebralis is an important insect neuroendocrine centre, composed of neurosecretory cells that regulate feeding

(olfactory/gustatory perception of food sources; feedback information from the intestinal tract and body cavity regarding the urgency of feeding) and reproductive behaviours (de Velasco et al., 2007). *TAR1*^{PL00408} flies showed a phenotypic profile that correlates with these observations. These flies are in fact characterized by increased body fat, higher food intake and starvation resistance as well as reduced locomotor activity and metabolic rate in comparison to y^1w^{1118} controls (Li et al., 2016, 2017). These metabolic alterations were not sex dependent, although the effects in *TAR1*^{PL00408} males appeared to be more pronounced as compared with those seen in females. This could be related to sex-dependent differences in TAR1 expression, the mRNA of which accumulated at higher levels in males than in females (Finetti et al., 2020). Despite all this, little is still known about the precise mechanism by which the tyraminergic system modulates essential metabolic traits such as fat body, food intake, starvation resistance, locomotor activity and metabolic rate.

In insects, fat is mainly stored in the fat body, which is also one of the most important metabolic centres (Arrese and Soulages, 2010). Lipid storage and release are mainly controlled by two hormones, the *Drosophila* insulin-like peptides (mainly dILP2) and adipokinetic hormone (AKH, analogous to mammalian glucagon) (Roeder, 2020). During an acute stress situation, the mobilization of lipids is essential for survival. This mechanism appears to be also controlled by both OA and TA, presumably through modulation of dILP secretion (Fields and Woodring, 1991; Orchard et al., 1993). In fact, it has recently been observed that in *Caenorhabditis elegans*, during acute stress, TA accumulates, which in turn modulates the insulin signal (De Rosa et al., 2019). Therefore, the increased TG level observed in *TAR1*^{PL00408}, as compared with y^1w^{1118} control flies, might be related to a direct tyraminergic action on the release of dILPs. RNAi-mediated TAR1 silencing, targeted to the fat body, triggered a reduction of dILP2 in insulin-producing cells in the *D. melanogaster* pars intercerebralis and an increase in TG accumulation (Li et al., 2017). The increased TG levels in *TAR1*^{PL00408} flies could also be linked to enhanced food intake as well as to lower movement propensity and metabolic rate. It has recently been proposed, in fact, that TAR1 could be involved in processes related to sugar sensibility and food intake regulation (Ishida and Ozaki, 2011). For example, *honoka* flies showed a reduced sugar response (Damrau et al., 2018) linked to differences in food intake. It is worth noting that TAR1 is highly expressed in neurons located in the suboesophageal ganglia that are presumably associated with the salivary glands and neck muscle control, and are thus linked with feeding.

After monoterpene treatment, both *D. melanogaster* y^1w^{1118} and *D. suzukii* showed alterations in all behavioural assays performed. The link between monoterpene treatment and *TAR1* downregulation is supported by the higher food intake observed in response to this treatment. When the *D. melanogaster TAR1*^{PL00408} deficient line was considered, no phenotypic changes were observed whatsoever after exposure to monoterpenes, suggesting that the alterations observed in the other genotypes require the correct expression of a functioning receptor. This further confirms the relationship between monoterpene-induced behavioural changes and TAR1. TAR1-mediated physiological alterations due to monoterpenes were also observed in *Phormia regina*. In fact, D-limonene treatment decreased TA levels in *P. regina* brain, causing a direct modification of food intake (Nishimura et al., 2005). This different response to food stimuli was subsequently attributed to a probable alteration of TAR1 expression at the level of the suboesophageal ganglion (Ishida and Ozaki, 2011). Furthermore, thymol and carvacrol appeared to play a crucial role modulating ant behaviour (locomotion and aggression), through aminergic regulation (Mannino et al., 2018).

In conclusion, this study shows that monoterpenes might be instrumental in the manipulation of the insect behaviour via TAR1. In fact, sublethal concentrations of thymol, carvacrol and α-terpineol downregulate *TAR1* expression, ultimately affecting important metabolic traits such as starvation resistance and energy storage. Moreover, this work demonstrates that monoterpenes, in addition to their insecticidal properties, can modify the metabolism and fitness of surviving *D. suzukii*, opening the way to innovative applications of these molecules in pest control.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: G.B., T.R.; Methodology: L.F., L.T., X.Z., S.C.; Data curation: L.T., L.F.; Writing - original draft: L.F.; Writing - review & editing: G.B., T.R.; Supervision: G.B., T.R.; Project administration: G.B., T.R.

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